2019

Endotoxin Increases Oxidative Stress And Oxygen Tension While Reducing Milk Protein Gene Expression In The Mammary Gland

Alexander Jonathan Spitzer
University of Vermont

ENDOTOXIN INCREASES OXIDATIVE STRESS AND OXYGEN TENSION WHILE REDUCING MILK PROTEIN GENE EXPRESSION IN THE MAMMARY GLAND

A Thesis Presented

by

Alexander Jonathan Spitzer

to

The Faculty of the Graduate College

of

The University of Vermont

In Partial Fulfillment of the Requirements
for the Degree of Master of Science
Specializing in Animal Science

August, 2019

Defense Date: May 9, 2019
Thesis Examination Committee:

Feng-Qi Zhao, Ph.D., Advisor
Douglas I. Johnson, Ph.D., Chairperson
David H. Townson, Ph.D.
Cynthia J. Forehand, Ph.D., Dean of the Graduate College

ABSTRACT

Mastitis, the inflammation of the mammary gland by bacterial infection, is one of the costliest diseases to the dairy industry primarily due to a loss in milk production. The aim of this study was to investigate the mechanisms underlying reduced milk production during mastitis. We hypothesized that bacterial endotoxin induces cell apoptosis, oxidative stress and increases hypoxia while inhibiting milk gene expression in the mammary gland. To test this hypothesis, mice were bred to pregnancy, and 3 days post-partum the left and right sides of the 4th pair of mammary glands were alternately injected with either the endotoxin liposaccharide (LPS, E. coli 055:B5, 100 ul of 0.2 mg/ml) or sterile PBS through the teat meatus. At 10.5 and 22.5 h post-injection, pimonidazole HCl, a hypoxyprobe, was injected intraperitoneally. At 12 or 24 h after the LPS injection, the fourth glands were individually collected (n=8 pairs) and analyzed for hypoxia, gene expression and oxidative stress. LPS treatment induced mammary gland inflammation as shown by increases in inflammatory cytokine expression ($P < 0.001$) and neutrophil recruitment at 12 and 24 h. LPS promoted cell apoptosis in a transient manner; an abundance of cleaved caspase 3 was evident only at 12 h after LPS challenge ($P = 0.02$). Increased H2O2 content was seen at 12 h ($P < 0.001$) but decreased dramatically after 24 h of LPS treatment ($P < 0.001$). Total antioxidative capacity tended to decrease at both 12 and 24 h ($P = 0.067$ and 0.061, respectively). In agreement with these findings, LPS activated Nuclear factor erythroid 2-related factor 2 (Nrf2) antioxidative signaling in the mammary gland, demonstrated by increased expression of its target gene Nqo1 at 12 h ($P = 0.05$) and xCT at 24 h ($P = 0.076$). Hypoxyprobe staining, indicative of hypoxia, was greater in the alveoli of PBS-treated glands than LPS-treated glands at both 12 and 24 h. This suggests oxygen tension rises in response to LPS treatment. Conversely, milk expression genes, β-casein gene (CSN2) and α-lactalbumin (LALBA), were inhibited by LPS treatment across time. Expression of α-S1 casein (CSN1S1) mRNA increased with LPS treatment at 24 h, but protein expression was reduced at this same time point ($P < 0.05$). In summary, intramammary LPS challenge incurs inflammation, augments cell apoptosis, induces oxidative stress and activation of the Nrf2 antioxidation pathway, increases oxygen tension, and inhibits milk protein expression in the mammary gland. This study provides functional insight into mechanisms of reduced milk production during mastitis and provides possible approaches to combat reduction in milk production, such as enhancing the Nrf2-antioxidative signaling pathway and reducing inhibition of milk protein expression.

Keywords: Antioxidation, Gene expression, Hypoxia, Inflammation, Mastitis, Nrf2

ACKNOWLEDGMENTS

Here I would like to acknowledge who have helped me during my Graduate studies at the Department of Animal and Veterinary Sciences, especially my advisor Dr. Feng-Qi Zhao. Feng-Qi always pushed me to strive for the best quality of my lab work, writing, teaching and as a person professionally and in general. I have known him since my undergraduate study and could not think of a better mentor. I am also grateful for my committee chair Dr. Doug Johnson, who taught my first microbiology course and encouraged me to develop an interest in infectious diseases. I am also appreciative of Dr. Dave Townson for serving on my committee and being able to give suggestions and advice as I progressed through my research and the graduate program. I am grateful for the help I received from Dr. Alan Howard, the esteemed members of the Microscopy and Imaging Center, and Dr. Scott Tighe and the proud members of the Vermont Cancer Center. My lab members Dr. Ratan Choudhary, Miyuki Takashima, Qing Tian, Dina Alter, and Leslie Rivers in addition to other peers and friends have been very supportive and were able to go above and beyond to help me achieve my goals for my studies in addition to making my time a unique and pleasurable experience.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 1: Literature Review

1.1. Introduction

Mastitis, one of the costliest diseases in the dairy industry, is inflammation of the mammary gland commonly caused by intramammary bacterial infection. Bacterial invasion of the mammary gland through the streak canal allows such organisms to proliferate on nutritious milk components, such as lactose and caseins (Zhao *et al*., 2008). Macrophages and other innate immune cells produce inflammatory mediators that recruit polymorphonuclear neutrophils (PMNs) and other immune cells towards the infected areas. These cells cross tight junctions of mammary epithelial cells (MECs) from the stromal tissues and enter the alveolar lumen, which stimulate the inflammatory hallmarks of heat, pain, redness, and swelling in the mammary gland (Zhao *et al*., 2008). This, however, is not seen in subclinical mastitis, which accounts for most mastitis cases annually (Zhao *et al*., 2008), but milk production is also negatively affected in subclinical mastitis. Mastitis also results in increased pH, sodium and chloride ion content, and somatic cell count in milk that ultimately force farmers to discard this low-quality milk (Ogola *et al*., 2007). The loss in milk production is the primary economic cost of mastitis with increased veterinary care and other factors totaling annual losses at $2 billion (Suszkiw, 2002). The specific mechanisms of how mastitis inhibits milk synthesis of the mammary gland are not clear, thus, we aim to determine the potential reasons for the loss in milk production by mastitis. In this review, our current knowledge in mammary development, milk protein and lactose synthesis, the pathophysiology of mastitis, and the role of oxidative stress and hypoxia in mastitis are introduced.

1.2. Mammary Gland Development and Lactogenesis

The mammary gland is a dynamic organ that develops throughout the life of mammals. This skin organ, upon maturity of the mammal, undergoes cyclical changes in four stages: mammogenesis, lactogenesis, galactopoiesis, and involution. The actions of MECs, stromal cells, and immune cells work in concert with reproductive hormones, metabolic hormones, and locally produced factors to accomplish this feat. This section discusses the stages of mammary development and the intricate processes that permit these changes to proceed in a coordinated manner.

1.2.1. Mammogenesis

The parenchymal tissue of the mammary gland is developed by the process of mammogenesis in all mammals. Long before the mammary gland produces milk, it undergoes significant changes in 3 major phases: fetal, pubertal, and reproductive.

Fetal Development

During early development, the ectoderm of the embryo gives rise to all epithelial tissue while the fat pad and all other stromal tissue stems from the mesoderm. In mice, fetal mammary gland development commences with the mammary band, a line of ectodermal cells that will become the parenchyma (Veltmaat *et al*., 2003). The principal activity occurring in this phase is the invasion of the ectodermal cells into the mesenchymal layer, which eventually forms a divot on the embryo. It starts at day 10 of embryogenesis in mice and day 32 in cattle with the mammary line, the structure which expresses increased Wnt signaling relative to other tissues (Veltmaat *et al*., 2003; Akers, 2002). The next step is the formation of the mammary gland, which is aided by fibroblast precursors of the mesenchyme that secrete fibroblast growth factor (FGF). FGF10

promotes Wnt10b signaling, and FGF receptor knockout mice lack placode development (Dzięgelewska *et al*., 2018; Mailleux *et al*., 2002). In mice, the assembly of 5 pairs of mammary placodes occurs by embryonic day 11 (Cowin *et al*., 2010). The invasion by the ectoderm into the mesoderm forms buds at day 14 in females (Richert *et al*., 2000). In males, the testes secrete androgens on day 13 and inhibit the developmental process with the precursor gland sloughing off by day 15 (Richert *et al*., 2000).

Following these phases is when the mesenchymal layer grows to extend a mammary bud from the body to form the primary sprout, the progenitor to the gland cistern in cattle or the lactiferous sinus in humans and mice. More ectodermal cells expand into the fat pad forming solid clusters of epithelia surrounded by minute layer of fibroblast cells. Once this primary sprout reaches the fat pad precursor made up of preadipocytes, it divides congruently to ultimately yield 10-15 branches known as secondary sprouts in mice (Elo *et al*., 2017). In mice, the primary sprout is formed on day 16 followed by the secondary sprouts on day 17 branching in the mammary gland (Richert *et al*., 2000). In cattle, formation of the primary sprout occurs around day 80 of embryogenesis (Akers, 2002). To create a lumen within the epithelium, the canalization of the mammary gland is initiated at the primary sprout. Canalization then proceeds towards the secondary sprouts to form the gland cistern and ducts, respectively, as well as towards the external surface to form the teat. Parathyroid-related hormone is endogenously secreted in this tissue to act as a critical mediator of the phase shift from bud formation to branching (Wysolmerski *et al*., 1998). The embryonic development is completed by day 18.5 in mice and will remain quiescent until puberty and the mammary

gland grows isometrically, the same rate as the rest of the body, during this period (Sakakura, 1987).

Puberty

The mammary gland is a unique organ that undergoes cyclical growth and functional differentiation following puberty. The pubertal stage of mammogenesis is initiated following the stimuli of hormones and other controls expressed at the onset of puberty. Primarily, the secretion of follicle-stimulating hormone and luteinizing hormone from the anterior pituitary in a cyclical manner stimulates the secretion of estrogen and progesterone from the ovary. Estrogen acts synergistically with growth hormone and prolactin from the anterior pituitary to stimulate ductal branching and elongation, which drives the allometric growth of the mammary gland. Allometric growth refers to a tissue that grows at a rate faster than the rest of the body. This contrasts with isometric growth. Estrogen mediated ductal morphogenesis occurs through estrogen receptor alpha, which is integrally controlled in part by forkhead box protein A1 (Liu *et al*., 2016). Progesterone, meanwhile, causes ductal cell proliferation, leading to the enlargement and development of ductal side branching (Atwood *et al*., 2000). These hormones together increase mammary epithelial growth tremendously alongside somatotropin, whose effects may be largely mediated by increased insulin-like growth factor 1 (IGF-1) among other paracrine signals. IGF-1 promotes cell growth, differentiation, and prevents apoptosis. In mice and other rodents, paracrine signals like increased IGF-1 causes terminal end buds to disseminate throughout the mammary fat pad. The terminal end bud structure in the mouse mammary gland contains a single layer of cap epithelial cells containing numerous stratified body epithelial cells that become myoepithelial cells and luminal epithelial cells

respectively (Williams and Daniel, 1983). Terminal end bud formation is due in part to FGF signaling because FGF20 knockout mice have delayed invasion by ducts during puberty (Elo *et al*., 2017). In ruminants and human, there are no terminal end buds, but rather terminal ductal lobular units (TDLU). These are considered the developmental unit of the mammary gland and have a similar bilayered structure of basal cells and luminal cells that become myoepithelial cells and epithelial cells, respectively (Dzięgelewska *et al*., 2018). Progesterone stimulates secretion of transforming-growth factor beta (TGFβ), which inhibits alveolar growth and prevents milk production (Kim *et al*., 2005). While the epithelial component of mammary tissue is similar across mammals, the stromal composition varies greatly from species to species. Mice possess mostly white adipose tissue with little fibrous connective tissue. Conversely, human and bovine mammary tissue possesses more fibronectin and other fibrous connective tissue (Dzięgelewska *et al*., 2018). Either way, the stromal tissue plays a vital role in mammary development.

Stromal tissue can promote mammary growth via the production of TGF-alpha, epidermal growth factor (EGF), and FGF by fibroblasts around terminal end buds following activation by estrogen and somatotropin (Unsworth *et al*., 2014). FGF2 signaling inhibitor Spry2-knockout mice have increased elongation and invasion into the fat pad during puberty while overexpression of Spry2 represses ductal morphogenesis (Dzięgelewska *et al*., 2018). Adipose is necessary for the formation of an advanced ductal network in puberty as well as in adulthood because the adipose provides signals to the growing epithelium. CC chemokine ligand 2 and pro-inflammatory ERK1/2 signaling promote estrogen biosynthesis in the mammary adipose tissue via increased aromatase

expression (Martínez-Chacón *et al*., 2019). Adipose specific vitamin D receptor knockout mice fed a high fat diet have enhanced ductal morphogenesis despite no difference in fat pad mass (Matthews *et al*., 2016) As this ductal network expands, so does the mammary vasculature in a proportional manner to provide vital nutrients to the growing branches (Yu *et al*., 2017).

In addition to stromal tissue, the immune system assists in the elongation and dissemination of the terminal end bud. Mast cells are one of the first immune cells to arrive to the TEB or TDLU in mice and cattle, respectively, and promote the extension of ducts into the mammary fat pad (Beaudry *et al*., 2015). Interleukin-5 (IL-5) deficiency reduces morphogenesis and pup survival (Dzięgelewska *et al*., 2018). Macrophages, meanwhile, are recruited to the mammary gland and aggregate around the TEB of mice or TDLU of cattle to promote collagen and stromal remodeling to pave the way for parenchymal growth (Beaudry *et al*., 2015). Following the pubertal stage, the mammary gland will not grow further until it is stimulated by cues unique to pregnancy.

1.2.2. Lactogenesis

Lactogenesis is considered the preparatory stage of the pregnant mammal's mammary gland for lactation and can be divided into 2 phases: lactogenesis I and lactogenesis II. Before lactogenesis occurs, the animal must first become pregnant, which elevates progesterone secretion along with a multitude of other factors. From here, lactogenesis I commences at around mid-gestation, which coincides with the start of exponential MEC growth and differentiation, including expression of milk synthesis genes. Luminal epithelial cells of the mammary gland are stimulated to proliferate and rapidly divide and form alveoli. The mammary alveolus, the secretory unit of the

mammary gland, is a structure containing a single layer of MECs enveloped by myoepithelial cells, capillaries, and other mesenchymal tissue. Adipocytes in the mammary gland decline to give way to cell proliferation in the formation of ducts and changes in stromal tissue such as angiogenesis and structural support for the developing secretory structure. Being an energy intensive process, the adipocytes become smaller as they provide additional energy for the increased metabolic activity (Gregor *et al*., 2013; Hovey and Aimo, 2010). The hormonal regulation of this development is species dependent.

Prolactin stimulates prolactin receptors, a type of tyrosine kinase of the Janus kinase family and mediates the phosphorylation and subsequent dimerization of signal transducer and activator of transcription 5a (*STAT*5a) with *STAT*5b (Buonfiglio *et al*., 2015). The dimerized *STATs* move into the nuclear compartment and stimulate expression of milk synthesis genes. *STAT*5 knockout mice have a decreased ability to produce milk as evidenced by decreased pup growth (Buonfiglio *et al*., 2015). Translation of milk synthesis gene mRNA is inhibited at this time, which may be a result of progesterone mediated expression of TGFβ (Kim *et al*., 2005).

When the mammal undergoes parturition, the mammary gland shifts from lactogenesis I to lactogenesis II as hormonal cues play a major role in this conversion. The hormonal activation of this shift is regulated by inhibitory progesterone and stimulatory prolactin. As progesterone sustains pregnancy, its secretion decreases at parturition and, along with a surge in prolactin secretion, stimulates the activation of milk synthesis. To assist in this production, the endoplasmic reticulum (ER) stress factors ATF4 and CHOP are upregulated to help manage the increased amount of stress placed

on the ER at this phase by protein synthesis (Yonekura *et al*., 2017). CHOP expression is not increased in mouse mammary tissue, which may be a result of lower total milk protein production relative to cattle (Yonekura *et al*., 2017). X-box binding protein 1 and ATF4, transcription factors of the unfolded protein response, are increased at this stage, which may result in increased cell differentiation via increased expression of glucocorticoid, insulin, and prolactin receptors (Tsuchiya *et al*., 2017).

In the mammary gland and the animal, there are several other changes that occur in the periparturient period. Upon completion of birth, the cardiac output directed towards the uterus is shifted to the mammary gland to support immense colostrogenesis and milk synthesis (Graćner *et al*., 2015). The cow enters a state of negative energy balance where her feed intake cannot match her metabolic demands, thus increasing non-esterified fatty acids (NEFA) and beta-hydroxybutyric acid concentrations in the bloodstream (Abdelli *et al*., 2017). The drop in progesterone coincides with rising mammary macrophage populations, activating NF-κβ signaling to increase tight junction permeability by cytokine signaling (Need *et al*., 2014). While there is no increase at the systemic level of thyroid hormone, the mammary gland expresses more thyroxine-5'-deiodinase to convert thyroxine to the more potent triiodothyroxine, which increases the metabolic capacity of mammary tissue at and through lactation (Campo Verde Arboccó *et al*., 2017). Since thyroxine-5'-deiodinase is somatotropin inducible, thyroid hormone activity is dictated by somatotropin, and hypothyroidism decreases *STAT*5 activation (Campo Verde Arboccó *et al*., 2017). The mRNA content of MECs increases 4-fold from late gestation to the start of lactation, and the energy and protein demand increases 5-fold during this

time as a result (Kuhn *et al*., 1999; Schingoethe *et al*., 1988). At this time, lactogenesis is achieved and the mammary gland becomes fully operational.

Galactopoiesis

Once lactation is initiated, it must be sustained by the process of galactopoiesis. The main stimulus to sustain this phase is milk letdown, and this lactation persistence is coordinated by endocrine and paracrine factors. In mice and most other mammals, prolactin is the primary galactopoietic hormone while somatotropin is the one in ruminants. Prolactin is released along with oxytocin in a neuroendocrine response as the hypothalamus coordinates their release from the anterior and posterior pituitary gland, respectively. Prolactin exerts its effect via binding with prolactin receptor and activating JAK-*STAT* signaling to stimulate further milk synthesis. Oxytocin, on the other hand, targets myoepithelial cells to contract causing milk ejection. In ruminants, somatotropin causes synthesis of downstream effector IGF-1 in the liver and locally in the mammary gland while acting in concert with oxytocin and prolactin (Akers, 2006). IGF-1 also promotes milk production in mice (Su, 2004). The exact role of somatotropin has yet to be elucidated, but its receptor expression is increased in lactating mammary glands (Sinowatz *et al*., 2000).

1.2.3. Mammary Involution and Apoptosis

Involution is the process by which the mammary gland shifts from galactopoiesis to a non-lactating state. In general, the process can be divided into a reversible first phase involving soluble factors and upregulation of the unfolded protein response followed by an irreversible second phase of protease-dependent structural changes to return the mammary gland to its quiescent state. The first period of involution is known as

reversible involution because milk removal will reverse any effects or signaling at this time. Without suckling or other milk removal methods, oxytocin and prolactin secretion is inhibited, thus reducing *STAT*5 activation. The main stimulus to induce involution is cessation of milk removal which inflicts the accumulation of feedback inhibitor of lactation (FIL), which can lead to irreversible involution if milk letdown does not occur frequently enough. The process of involution is species-dependent as it happens within 48 hours for mice, rats and other rodents, while it can be much longer for cattle. In cattle, milk protein gene expression remains unaltered until 3 days after weaning while expression of interleukin 1 beta converting enzyme, which cleaves pro-IL-1B to active IL-1B, increases within hours (Lund *et al*., 1996). As milk accumulates in the mammary gland so does IL-1B, TNF-alpha and other pro-inflammatory cytokines that promote cell death. Death receptor mediated apoptosis occurs through the release of cytochrome-c via inhibition of Bcl-2 by Janus Kinase to activate caspase 3. Cellular inhibitors of apoptosis proteins 1 and 2 prevent hyperactivation of caspase 3 and delay the cell death characteristic of involution (Carr *et al*., 2018). All these soluble factors can be alleviated by milk removal, thus emphasizing the importance of milk removal in the mammary gland.

Without milk removal, involution can become irreversible by entering the second stage of involution. Irreversible involution is characterized by clearance of secretory epithelial cells, extracellular matrix reconstruction, and repopulation by adipocytes. The second phase of involution culminates in the rigorous restructuring of the mammary gland through local cues communicating with the immune system and mesenchyme of the mammary gland. In ruminants, lactose synthesis decreases to lower milk volume and

cause involution. Milk protein content, on the other hand, increases with an elevated amount of lactoferrin, immunoglobulins while milk protein synthesis is reduced. Milk fat vacuoles accumulate in MECs and milk component synthesis is impaired. While reduction of milk biosynthesis is noteworthy in involution, there are 3 key events in irreversible involution: TGFβ production, *STAT*3 activation, and remodeling of the gland structure.

Irreversible involution coincides with the epithelial-mesenchymal transition and allows epithelial cells to lose their polarity and infiltrate the basement membrane. A secreted protein central to these processes is TGFβ, which is inhibited by lactogenic hormones and thus, increases in involution. TGFβ-1 promotes the growth of mesenchymal cells while reducing epithelial cell proliferation as evidenced by decreased milk synthesis and survival of MECs while promoting extracellular matrix deposition (Sudlow *et al*., 1994). In fact, TGFβ-1 induces Bax mediated apoptosis in the mouse MEC cell line, HC11 (Kolek *et al*., 2001). In this form of cell death, activated Bax is translocated to the mitochondria to cause cytochrome-c leakage and activates caspase-3 to trigger programmed-cell death in MECs. TGFβ-1 can also stimulate apoptosis by activating Smad2/3 transcription factors to promote expression of IGF binding proteins, which reduce free IGF concentrations. In bovine MEC cell line BME-UV1, IGF binding proteins prevent pro-survival signaling by the PI3K/Akt pathway and promote Bax-mediated apoptosis (Gajewska *et al*., 2004).

TGFβ-3 helps the process of involution by reducing tight junction and adherens junction integrity through activation of gamma-secretase, which cleaves E-cadherin and releases beta-catenin (Fornetti *et al*., 2015). Beta catenin then translocates to the nucleus

to stimulate MEC differentiation to a phagocytic cell (Fornetti *et al*., 2015). MEC differentiation for efferocytotic behavior is essential in proper involution. Deficiency of Mer tyrosine kinase, which inhibits pro-inflammatory signaling and promotes endocytosis in all phagocytic cells, impairs early processes of involution (Sandahl *et al*., 2010). TGFβ upregulates arachidonic acid synthesis, a precursor to inflammatory eicosanoids that are key signals to immune cells in this remodeling (Zhang *et al*., 2017). TGFβ-3 has an active role in involution as its expression stimulates phosphorylation of *STAT*3 within 8 hours of weaning (Nguyen, *et al*., 2000).

In contrast to reduced *STAT*5 at this stage, pro-apoptotic *STAT*3 increases to initiate the second, irreversible phase of involution (Uejyo *et al*., 2015). As cytokine production increases during involution, the tumor necrosis factor family of cytokines and other inflammatory mediators cause leukemia inhibitory factor synthesis and activation of *STAT*3 by Janus kinase (Quaglino *et al*., 2007). *STAT*3, stimulated by TGFβ-3, causes *STAT*3-dependent MEC expression of CD14, which is essential to phagocytic capability (Hughes *et al*., 2012). *STAT*3 activation promotes the expression of inhibitory subunits of PI3K to inhibit pro-survival signals by pAkt (Schuler *et al*., 2016). *STAT*3 also induces a phenotypic shift in macrophages from M1 to M2 (Hughes *et al*., 2012). Dendritic cells are the first immune cell to appear in large numbers, which coincides with signaling similar to wound healing (Martinson *et al*., 2014). Immunomodulatory signaling seen in wound healing is critical to alter the macrophage function to increase their ability to reconstruct the connective tissue of the mammary gland.

*STAT*3 also mediates the production of CAAT/enhancer binding protein δ (C/EBP δ) and cathepsins B and L while inhibiting expression of Spi2a, a cathepsin

inhibitor, to encourage lysosomal mediated programmed cell death (LM-PCD) in MECs (Hughes *et al*., 2018). As apoptotic bodies and fat accumulate without milk removal, *STAT*3 coordinates MEC uptake of apoptotic bodies and milk fat globules, and the plasma membranes lose their fidelity to permit cathepsin release and mediates lysosomal mediated-programmed cell death (LM-PCD) (Sargeant *et al*., 2014). Milk fat globule EGF 8 catalyzes this process by tagging extracellular phosphatidylserine, a hallmark of apoptosis, on cellular debris and milk fat globules (Hanayama *et al*., 2005).

The stromal tissue increases in contrast to the decreasing luminal space, but structures do not atrophy in ruminants. In contrast, alveolar structures in rodents are abolished via cell death pathways. The primary cell death mechanism in rodents is apoptosis, which can be characterized as soon as 48 hours since last milking in addition to increased CD68 expression (Warri, *et al*., 2018). Remodeling of this tissue is encouraged by matrix metalloproteinases that digest the underlying connective tissue from the epithelium. Increased expression of proteolytic enzymes, such as stromelysin-1 and gelatinase-A secreted by some fibroblasts, is seen by day 4 of involution (Lund, *et al*., 1996). The proteolytic digestion of the basement membrane detaches MECs, resulting in their programmed-cell death. While the epithelial component is removed, the adipocytes, which are no longer supplying massive amounts of energy for anabolism, repopulate the mammary gland 4.1-fold relative to lactation and increase their fatty acid content, therefore, cells are increasing in size (Zwick *et al*., 2018).

At the completion of mammary involution, the gland is ready to begin the cycle once again. Through these cyclical developmental stages, the mammary gland can gain more and more parenchymal tissue at the end of each cycle so that the next cycle will be

more productive. This intricate biology is widely studied, but many aspects of mammary development remain elusive, such as the regulation of milk protein synthesis and the effect of mastitis among other factors.

1.3. Milk Synthesis

The ultimate purpose of the mammary gland is to synthesize and secrete milk for the nourishment of the offspring. Milk is mainly comprised of proteins, carbohydrates, fats and water to provide the young with all essential nutrients for growth and health. While milk is a definitive characteristic of mammals, the composition varies greatly to best support the neonates in their indigenous habitat. Bovine milk contains 3.2% protein, 4.8% lactose, and 3.5% fat on average while human milk is 1.1%, 6.8% and 4.5%, and rat milk is 11.3%, 2.9% and 14.8%, respectively (Vincent *et al*., 2016; Jensen, 1995). Despite differences across species, the synthesis of milk is relatively conserved. This section will focus on the characteristics and synthesis of milk proteins in addition to the production of lactose.

1.3.1. Milk protein synthesis

Milk Proteins

Milk proteins can be organized into casein proteins and whey proteins. Caseins comprise 80% of milk protein and include 4 major proteins: beta-casein (gene symbol: *CSN2*), alpha-S1 casein (*CSN1S1*), alpha-S2 casein (*CSN1S2*) and kappa-casein (*CSN3*) (Vincent, *et al*., 2016). These proteins are expressed in the bovine mammary gland at 38%, 35%, 10%, and 12%, respectively (Vincent *et al*., 2016). Caseins are ~20 kD phosphoproteins that attract calcium ions and condense to form micelles, which in turn provide micronutrients with highly digestible proteins to the neonate. The whey fraction,

18% of protein in bovine milk, is a heterogenous group and mainly includes alpha-lactalbumin (*LALBA*), serum albumin and immunoglobulins.

Milk proteins do not function solely for nutrition of the neonate. CSN2 has a high biological value and can exist in 13 variants and possess 4 to 5 phosphorylated residues (Vincent *et al*., 2016). CSN2 digestion produces biologically active peptides that have negative and positive effects on the body. A1 CSN2 variant, but not A2 CSN2 variant, increases the rate of type I diabetes in rats, and human type I diabetics have increased ratios of A1/A2 antibodies relative to controls (Elliot *et al*., 1997; Padberg *et al*., 1999). In a milk intolerance clinical study, it was found that those who consumed a non-dairy milk control containing A1 CSN2 reported more occurrences of severe digestive discomfort than those who consumed the A2 CSN2 analog (Ho *et al*., 2014). A2 CSN2 variants isolated from gouda cheese, on the other hand, have antihypertensive properties (Saito *et al*., 2003).

CSN1S1 also has antihypertensive properties (Saito *et al*., 2003). CSN1S1 is one of the major phosphoproteins found in milk that promote formation of colloidal micelles with calcium phosphate. CSN1S1 has 9 variants with 8 to 9 phosphorylated residues (Vincent *et al*., 2016). CSN1S1 is an activator of TLR4 signaling and subsequent pro-inflammatory cytokine signaling in human monocytes (Vordenbäumen *et al*., 2016). These results were later confirmed as human recombinant CSN1S1 induced TLR4/MD2 receptor complex mediated IL-8 secretion from HEK293 cells (Saenger *et al*., 2019).

Transcriptional Regulation

In protein synthesis, a gene must be first transcribed from DNA to RNA. Milk protein gene transcription is cis-regulated through transcription factor mediated effects of

multiple hormones, such as insulin, prolactin, and glucocorticoids (lactogenic hormone complex, HIP). Being a conserved process across most casein proteins, the CSN2 gene is widely utilized as a model for studying milk protein gene expression (Jones *et al*., 1985). Approximately 250 bp upstream from the CSN2 gene transcription start site (TSS) is its proximal promoter that is preceded by the distal enhancers -1.6 to -6 kb upstream of the TSS depending on species (Qian *et al*., 2014). The transcription factors that target the promoter include *STAT*5, C/EBPβ, and the repressor Yin Yang 1 (YY-1) along with the glucocorticoid receptor, octamer binding factor-1 (Oct-1), and runt-related transcription factor 2 (Runx2) (Qian *et al*., 2014). The distal enhancer of the CSN2 gene exerts its action in combination with binding with these transcription factors too (Qian *et al*., 2014).

*STAT*5 is activated by prolactin via phosphorylation by Janus kinase (JAK). The action of *STAT*5 is amplified by glucocorticoid receptor, which increases efficiency of *STAT*5 phosphorylation and DNA binding (Pfitzner *et al*., 1998). *STAT*5a is a critical transcription factor of milk synthesis; *STAT*5a knockdown mice have decreased milk protein synthesis and reduced gap junction integrity (Reichstein *et al*., 2011). YY-1 is a weak repressor of milk protein expression that is easily dislodged by *STAT*5 and C/EBPβ binding at the CSN2 promoter (Wyszomierski *et al*., 2001).

While prolactin and glucocorticoids work together to stimulate milk protein synthesis, insulin also plays a key role. It may work through PI3K/Akt signaling pathway as this stimulates expression of the enhancer E74-like factor 5 (ELF5) that binds to the CSN2 proximal promoter (Lemay *et al*., 2007). While Oct-1 can act with progesterone receptor to inhibit CSN2 expression during pregnancy, Runx2 can bind with Oct-1 to

enhance transcription of CSN2 (Inman *et al*., 2005; Qian *et al*., 2013). In a coordinated manner, these transcription factors synergize to stimulate milk protein mRNA synthesis. Following transcription, the mRNA is utilized as a blueprint to build proteins.

Translational Regulation

After transcription, the primary transcript (pre-mRNA) moves from the nucleus of the cells to the rough ER to be translated by ribosomes. Before translation, the pre-mRNA undergoes modifications, such as the removal of introns to leave the 9 exons (mature mRNA) of the CSN2 gene (Jones *et al*., 1985). Translation of milk protein mRNA is an involved process that depends on amino acid availability, accessible energy content, and signaling of the principal translational regulator, the mTOR pathway. Together, these processes work in concert to translate milk protein mRNA in a carefully ordered manner.

In order to produce milk protein from mRNA via translation, amino acids must be supplied to the MECs. This is done with amino acid transporters, which transfer all essential amino acids and partial non-essential amino acids from the extracellular fluid into the cells. Amino acid transporters found in MECs include sodium dependent and sodium independent transporters and both differ in function and specificity of amino acids transported. Sodium-dependent transporters deliver glutamate, taurine and anionic amino acids in conjunction with sodium transport whereas sodium-independent transport targets neutral and cationic amino acids (Shennan *et al*., 1997). The rest of non-essential amino acids can be synthesized by the cell. Amino acid supplementation is positively correlated with bovine milk protein content (Lin *et al*., 2018).

The availability of energy is essential for protein synthesis like any other anabolic process. In the case of protein synthesis, the need for energy is great as the animal shifts into negative energy balance at parturition and sustains this state in early lactation (Gross *et al*., 2011). In fact, cows on energy restricted diets have decreased milk yield with lower protein content in as little as one week (Gross *et al*., 2011). What is interesting is that increased starch ingestion raises milk protein synthesis more than supplemental protein by increasing mammary blood flow and reducing amino acid catabolism (Rius *et al*., 2010). By transcriptome analysis, it was found that overall mammary gland gene expression of metabolic components is increased in lactation, including amino acid transaminases to supplement the Krebs cycle while glucose is directed to lactose synthesis rather than energy production (Bionaz *et al*., 2012). Energy status of the MECs controls mTOR signaling through activation of AMPK. Low cellular ATP concentration leads to increased AMPK phosphorylation to reduce mTOR activation and increase eukaryotic elongation factor-2 kinase (eEF2K) phosphorylation to inhibit translation (Appuhamy *et al*., 2014).

The mTOR pathway is the main pathway that regulates the translation of milk protein genes in accordance with nutrient availability and cues in the mammary gland. It serves to phosphorylate eukaryotic translation initiation factor 4E (eIF4E) binding protein 1 to activate eIF4E mediated translation initiation complex formation (Ma *et al*., 2009). It also deactivates eEF2K to activate its target and promotes ribosomal protein S6 to increase overall translational efficiency (Ma *et al*., 2009). To prevent inappropriate mTOR activation, amino acid sensing is tightly coordinated with these translational

machineries. Amino acid sensors like arginine sensor SLC38A9 activate Rag-GTPase regulator complex, which is responsible for mTOR activation (Wyant *et al*., 2017).

Nutrient availability, however, is not the only factor in the PI3K/Akt/mTOR pathway. The lactogenic hormone complex of HIP upregulates mTOR signaling greater than supplementary amino acids, glucose and acetate in bovine-derived mammary acini via Akt activation (Burgos *et al*., 2010). Both insulin and prolactin mediated signaling reduces activated menin, which is a negative upstream regulator of mTOR activity via inhibition of PI3K activation of Akt (Li *et al*., 2017). Insulin promotes translation of CSN2 mRNA by recruiting cytoplasmic polyadenylation element binding proteins to lengthen the poly-A tail and drives forward the initiation of translation (Choi *et al*., 2004). Prolactin activates JNK mediated tudor staphylococcal nuclease translocation to the nucleus, which stimulates mTOR signaling and CSN2 synthesis (Zhang *et al*., 2019).

Bovine and swine mammary tissue presented evidence for somatotropin as an activator of the mTOR pathway and subsequent mRNA translation, initiation and elongation (Hayashi *et al*., 2007; Burgos *et al*., 2010). Somatotropin increases 4E-BP1, eEF2K, and ribosomal protein S6 phosphorylation to enhance initiation and elongation of translation (Hayashi *et al*., 2009). The effects of somatotropin are potentially mediated by IGF-1, which activates mTORC1 kinase activity (Burgos *et al*., 2010). Annexin A2, a calcium dependent phospholipid binding protein, upregulates milk synthesis and MEC proliferation by increasing PI3K-Akt mediated mTOR activation and cyclin D1 expression (Zhang *et al*., 2018).

Following translation, caseins proteins must be modified by specific kinases to become phosphoproteins. Caseins are transported from the rough ER to the Golgi

apparatus to receive post-translational modifications. The main sites of casein phosphorylation are serine and threonine residues with CSN2 being phosphorylated on 4 to 5 residues and CSN1S1 on 8 to 9 residues (Farrell *et al*., 2004). The CSN1S1 8 phosphorylation residue isoform is expressed 3-fold greater than the 9-residue isoform and, while it is known that Golgi-enriched fraction casein kinase phosphorylates the 8-residue isoform and most other caseins, the kinase responsible for the 9th phosphorylation is unknown (Bijl *et al*., 2014). While many things are known about milk protein synthesis, the mysteries surrounding it remain abundant.

1.3.2. Lactose Synthesis

Glucose Uptake

Simple sugars, such as glucose, provide energy to cells for metabolic and biosynthetic purposes. In the MECs, glucose is also the major precursor for lactose synthesis (Zhao, 2014). MECs express multiple facilitative glucose transporters (GLUTs) that facilitate the uptake of glucose and other monosaccharides in a closely monitored manner (Zhao and Keating, 2012). Glucose uptake is driven by hexokinase phosphorylation to maintain a concentration gradient. In MECs, most of the glucose transporters face the basolateral membrane as glucose comes from the bloodstream, thus flowing down its concentration gradient (Brun del Re *et al*., 2010). GLUT1 is the primary transporter found here and plays a major role in glucose uptake in MECs (Zhao *et al*., 2007). The concentration of glucose within the cell is approximately the same as that of the milk (Kuhn *et al*., 1975). To generate lactose, glucose must be transported into the Golgi apparatus via these transporters. Prolactin causes GLUT1 subcellular localization to the Golgi apparatus membrane to facilitate glucose transport into the Golgi apparatus

for glycosylation and lactose synthesis (Riskin *et al*., 2015). In addition to GLUTs, MECs express sodium dependent glucose cotransporters (SGLT1 and SGLT2) (Zhao, 2014), but their physiological function is not known. In bovine mammary glands, there is a sharp rise in GLUT1 expression with less dramatic increases in GLUT8, GLUT12 and SGLT1 to coincide with increased glucose uptake from late gestation to early lactation (Zhao *et al*., 2007). While this trend is observed in different species, it remains unclear whether lactogenic hormone complex HIP or somatotropin mediates these increases or some other factors (Shao *et al*., 2013, Zhao *et al*., 1996). What is known, however, is that GLUT4 expression is decreased in the mammary gland during this period, which coincides with a decrease in adipose tissue and a decrease in insulin sensitivity during lactation (Zhao *et al*., 1996).

While galactose can be transported by GLUT for its uptake, the MECs can synthesize their own. By generating glucose 6 phosphate, MECs can synthesize galactose by a series of enzymatic reactions. Uridine diphosphate-glucose is generated from glucose and uridine triphosphate and then, via UDP-Galactose-4 epimerase, UDP-glucose is reconfigured into UDP-galactose (Sprong *et al*., 2003). UDP-galactose is transferred to the Golgi apparatus where it is combined with free glucose to form lactose (Neville *et al*., 2018).

Beta-Galactosyltransferase

Lactose synthase is the enzyme responsible for the condensation reaction that forms lactose. Lactose synthase is a heterodimeric enzyme, consisting of beta 1-4 galactosyltransferase-1 (b4GALT1) and regulatory subunit LALBA. b4GALT1 is a constitutively expressed enzyme in most cells found along the interior of the Golgi

apparatus (Smith *et al*., 1977). The isoform found in the Golgi apparatus of MECs is responsible for catalyzing the formation of D-galactosyl-N-Acetyl-D-Glucosamine in a non-lactation state, which is its normal function across tissues (Smith *et al*., 1977). b4GALT1 utilizes UDP-galactose to condense with N-Acetyl-D-glucosamine (Glc-NAc) in formation of galactosylated glycoproteins (Smith *et al*., 1977). About halfway through pregnancy, the start of lactogenesis I, this enzyme's substrate switches to lactose formation by acquiring an affinity for D-glucose rather than Glc-NAc (Smith *et al*., 1977). The expression of b4GALT1 is increased 2.5-fold from mid-gestation to early lactation in sows (Chen *et al*., 2017).

Alpha-Lactalbumin

b4GALT1 forms a heterodimer with LALBA to form fully functional lactose synthase. LALBA comprises 25% of the total protein content in human milk and a quarter of the bovine whey fraction, which amounts to 3% of the bovine milk protein (Holm *et al*., 2018). The expression of LALBA starts at lactogenesis I and continues until the end of lactation, and LALBA increases the binding affinity of b4GALT1 for glucose 1000-fold relative to b4GALT1 alone (Nicoleta *et al*., 2010). Being a carbohydrate, lactose cannot diffuse out of the Golgi apparatus and is instead secreted into the alveolar lumen with milk proteins. Because of this, water is recruited to the Golgi and secretory vesicles and, therefore, the Golgi and secretory vesicles are round and expanded in lactating MECs. While lactose synthesis is the primary biological function of LALBA, this protein can fulfill other duties. It also binds calcium, cobalt, and zinc ions in the whey fraction of milk (Nicoleta *et al*., 2010), and prevents coagulation of bovine milk proteins in a synergistic manner with CSN2 (Marciniak *et al*., 2018).

Lactose and protein synthesis contribute a major amount of nutrition derived from milk. The milk composition of mammals is species dependent, but the principal machinery in milk synthesis remains relatively similar. The end-product of this synthesis, milk, is a desirable food source for neonates and mankind.

1.4. Mastitis

The high nutrient content of milk makes it desirable to not only mammals, but bacteria as well. The lactating mammary gland is often the site of bacterial invasion and infections that result in mastitis. Bacterial infection is almost the sole cause of mastitis in cows, but the types of mastitis are varied based on the source and species of these etiological agents and the virulence factors presented by these bacteria.

Inflammation is defined by the four characteristics of heat, pain, redness and swelling, and the mammary gland is no exception to this. Mastitis causes not only physiological changes, but it can also create physical changes in the gland's appearance and function. The invading pathogens activate the innate immune system, which is responsible for these changes such as a rise in temperature to promote immune function. The inflammatory signaling by the innate immune system causes nerve cells to sensitize and blood vessels to dilate, thus causing the skin to become more tender and red. This vasodilation is accompanied by breaches in the tight junction of MECs to create a route for immune cell migration. As a result, milk quality suffers as milk production decreases and sodium, serum and immune components from the extracellular fluid flood the mammary lumen to displace the protein production. This milk also contains very little lactose and high amounts of somatic cells as the infection progresses. The ultimate result

of the immune action in mastitis is a vastly decreased milk yield with low quality product that is unfit for human consumption.

1.4.1. Types of Mastitis

While mastitis results in poor milk production and quality, the severity of infection varies greatly from case to case. Mastitis that causes clinical symptoms is known as clinical mastitis whereas mastitis presenting few if any symptoms is considered subclinical. Clinical mastitis is the most severe of these 2 types and is more lethal as its acute infections present severe local inflammation with sepsis-induced fever and other systemic effects. Endotoxemia is often a critical condition developed in prolonged clinical mastitis (Peters *et al*., 2015). Unlike clinical mastitis, subclinical mastitis is often asymptomatic and goes unnoticed, resulting in more chronic infections (Peters *et al*., 2015). Mastitis can be evaluated based on the milk yield, electrolyte content and somatic cell count. Severity of mastitis increases with somatic cell count and electrolyte concentration while milk yield presents an inverse relationship and is more and more repressed as the condition worsens (Bagri *et al*., 2018).

Mastitis can also be divided into 2 subclasses based on the source of infection: environmental mastitis and contagious mastitis. These two types of mastitis have key differences in the mode of transmission and the bacterial species behind mammary infections. Environmental mastitis occurs more often during the summer when heat stimulates bacterial growth in water, feces, bedding, mud and other areas the cattle may meet. Flies can carry coliforms from fecal matter and hardy Gram-Positive bacteria. The etiological agents behind this type of mastitis can potentially include *Escherichia coli* (*E. coli*)*, Streptococci, and Pseudomonas spp.*

Contagious mastitis is spread from cow to cow through contact with contaminated skin or sponges and towels used to wash the udder, or from calf to cow via suckling. Milking can cause milk fomites to disperse into the environment or to contaminate the milking equipment. Etiological agents for contagious mastitis include Gram-Positive cocci *Staphylococcus aureus* (*S. aureus*) *and Streptococcus agalactiae* (*S. agalactiae*) along with *Mycoplasma bovis* (*M. bovis*). These are often commensal organisms on human skin and cannot thrive in an environment separate from the host. These infections commonly result in subclinical and chronic infections, especially *S. aureus*.

1.4.2. Bacterial Pathogens

As stated earlier, mastitis is often the result of bacterial infection. Because mastitis is caused by mostly *S. aureus, streptococci*, *M. bovis* and Gram-Negative bacteria, the infections that stimulate inflammatory responses are coordinated in part by the nature of the pathogen and their surrounding environment (Cote-Gravel and Malouin, 2019). In this section, the pathogens most commonly associated with mastitis, listed above, will be discussed with their virulence factors and how they impact this infectious disease.

Staphylococcus aureus: In the dairy industry, *S. aureus* is considered one of the most commonly found etiological agents of mastitis and is of significant interest in medicine. *S. aureus* is a Gram-Positive coccus that displays clustering formation in Gram stains and are non-motile. They have many defining qualities among other *Staphylococcal* species, such as catalase positive and coagulase positive test results. They are desiccant resistant and halotolerant, which is a reason why they can successfully colonize the skin of humans and other mammals. *S. aureus* infection can be divided into

3 steps: adhesion to the ECM of the host, dissemination throughout the tissue and immune evasion (Middleton, 2008; Cote-Gravel and Malouin, 2019). *S. aureus* has many adhesins and binding proteins to assist it in anchoring itself and hiding from the immune system. The expression of adhesins in *S. aureus* is dictated by Sigma Factor B to increase adherence factors: collagen adhesin, fibronectin binding proteins A and B, teichoic acid of the cell wall, and microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) (Horsburgh *et al*., 2002; Otto, 2013).

As the cocci divide and replicate, the action of polysaccharide intercellular adhesin (PIA) allows *S. aureus* to bind to fellow microbes for biofilm formation and allow for evasion from neutrophil offenses (Sendi *et al*., 2009). When the growth rate is reduced towards stationary growth, the production of secreted exotoxins and other proteins is upregulated as autoinducing peptides (AIPs) activate sensor kinase AgrC to activate AgrA (Novick *et al*., 2008). AgrA upregulates small RNAs to promote exotoxin, protease and lipase synthesis and repress surface protein expression while AgrD codes for pro-AIPs (Novick *et al*., 2008). The breakdown of connective tissue by hyaluronidase allows *S. aureus* to infiltrate the stromal tissue of the mammary gland and potentially invade the capillaries and vessels.

To evade the immune system directly, *S. aureus* expresses numerous surface factors outside of PIA. Iron surface determinant A not only helps in sequestering iron from heme, but also binds fibrinogen and other serum proteins to the cell wall and form a protective clot (Clarke *et al*., 2009). These bacteria can release themselves from the clot by the action of Staphylokinase, an endogenous fibrinolysin, and this virulence factor can impair α-defensin and other soluble defense proteins (Jin *et al*., 2004). *S. aureus* is also

resistant to opsonization by immunoglobulins by the action of Staphylococcal protein A (SpA), which binds the conserved region of IgG antibodies that are prevalent in milk, along with staphylococcal binder of immunoglobulin (Serruto *et al*., 2010; Cole *et al*., 2016). *S. aureus* extracellular adherence protein binds ICAM-1 on endothelial cells along with fibrinogen to inhibit PMN adherence to endothelium and diapedesis. They can also avoid being captured by PMN NETosis with DNAses and can also inhibit phagosome-lysosome fusion in a SpA-mediated manner in vitro (McGuinness *et al*., 2016; Menegazzi *et al*., 2012).

S. aureus targets immune cells with a bevy of other toxins. From isolates of cattle with mastitis, *S. aureus* species secrete gamma hemolysins HlgAB and HlgCD, leukocidins (Luk) lukAB, lukED, and LukMF, and Panton-Valentine leukocidin (Vrieling *et al*., 2016). These pore-forming toxins specifically target cells expressing certain differentiation markers, like how LukED binds to CD195 (Alonzo *et al*., 2012). LukMF, but not LukAD, is the most potent leukocidin in bovine mastitis isolates (Vrieling *et al*., 2016).

While *S. aureus* possesses a plethora of virulence factors and other adaptations, it often lacks the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR associated system to protect against foreign genetic material. Because of this, *S. aureus* is very susceptible horizontal gene transfer, which is influenced by mobile genetic elements and can get genes to stimulate differentiation (Li and Zhao, 2018). In mastitis, MRSA ST398, a livestock associated strain, has been isolated more frequently in recent years and possesses SCCmec IV and V (Vanderhaeghen *et al*., 2010). This means that the

efficacy of the most commonly used antibiotics, beta-lactams, is reduced as this infectious disease adapts.

Streptococcus agalactiae: S. agalactiae are gram-positive diplococci of the group B streptococci and are non-motile, mesophilic, facultative anaerobes. This species is beta-hemolytic, which is essential for their function as they are auxotrophic for heme as well as for the L-isomer of many amino acids: arginine, cysteine, glutamic acid, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, tryptophan, tyrosine and valine (Glaser *et al*., 2002). This bacterium's reservoir is both in the host and in the environment. This species is often implicated in subclinical infections, and infected udders are a major reservoir. The disease can be transmitted by cow to cow transmission in udder washing and milking procedures. This species often infects during early lactation because of the immunosuppression by stress, low energy by low dry matter intake, and exposure to a milking machine that may be contaminated. *S. agalactiae* is a metabolically crippled bacterium implicated in numerous contagious and environmental mastitis cases.

Streptococcus uberis: *S. uberis* is another streptococci associated with more subclinical bovine mastitis cases, but does not have a streptococcal Lancefield grouping. It is an environmental pathogen found in manure or contaminated bedding and soil. It is also a commensal microbe and found along the body, the epidermis, genital tract, and tonsils of mammals, thus its prevalence fits its name. Although it is seemingly ubiquitous, *S. uberis* is auxotrophic for a variety of amino acids: glutamic acid, arginine, tryptophan, methionine, isoleucine, leucine, valine and histidine (Kitt *et al*., 1997). *S. uberis* commonly infects mammary gland during early involution, and these infections can often

continue into early lactation if not resolved. This species is considered a causative agent of subclinical mastitis more so than clinical mastitis.

Mycoplasma bovis: While not an issue in the United States, *M.* bovis causes contagious mastitis. *M. bovis* is not identifiable by gram staining due to a lack of peptidoglycan cell wall components found in either gram-negative or gram-positive bacteria. As a result of this, it is resistant to the antibiotics such as beta-lactams, bacitracin, and vancomycin. This bacterium is metabolically crippled and cannot produce its own ATP due to missing pieces of the oxidative phosphorylation pathway and lacks the capacity to operate the TCA cycle (Burki *et al*., 2017). To overcome these issues, it expresses numerous adhesins to bind to and invade the host cells for survival (Burki *et al*., 2017). They sometimes can create clinical mastitis but can also cause subclinical infections that make identification difficult. This is the most severe of all infections discussed because there is no cure for this infection and can disseminate quickly throughout a herd. The only certain way to contain the infection is to identify infected cows, separate them from the herd, which will usually lead to culling due to complete loss of production.

Escherichia coli and *Klebsiella pneumoniae* Gram-Negative rods: These bacteria are members of the Enterobacteriaceae family and are commonly found along the lower gastrointestinal tract of animals. These bacteria are implicated in more cases of environmental mastitis as their environmental reservoirs are manure and contaminated bedding. They are transmitted into the mammary gland by teat contact with contaminated materials or from milking procedures with suboptimal cleaning procedures. This type of infection is more likely to occur during the summer as the high temperatures provide

more favorable conditions for microbial growth, and heat stress is inflicted upon the cow that may stimulate immunosuppression by the hypothalamus-pituitary-adrenal axis. This is critical as one of their major endotoxins, lipopolysaccharide (LPS), initiates severe pro-inflammatory responses in a TLR4/MD2/CD14 coreceptor mediated manner that can result in febrile response, hypotension, and even death in cattle (Silanikove *et al*., 2011; Saenger *et al*., 2019).

1.4.3. Pathophysiology

Being a dynamic organ undergoing cyclical changes, the mammary gland is prone to diseases at certain points in the developmental cycle. Particularly, the periparturient period, early lactation, and involution are phases that leave the mammary gland at risk of infection. During the period shortly before and during calving, colostrogenesis takes place with IgG1 being the predominant antibody in addition to anti-LPS and other antibodies specific to bacterial toxins (Vilte *et al*., 2008). The colostrum also has little lactoferrin yet high citrate, which means there are fewer immune components to neutralize bacteria. Cows also begin to leak colostrum and milk components from teat ends during parturition due to pressure build up within the gland. This weakens the teat meatus and sphincter muscles and increases the likelihood of pathogen invasion.

Another time of increased risk of mastitis for dairy cows is early lactation. The cow enters early lactation where milk yield progressively increases and cows have a negative energy balance (Gross *et al*., 2011). An energy deficit results from decreased dry matter intake, a behavior that starts during the peripartum period. Body fat deposits are mobilized to meet the energy deficit (Abdelli *et al*., 2017), raising circulating beta-hydroxybutyrate and NEFA concentration. The rising of ketone bodies and NEFA in the

blood is immunosuppressive, causing decreased PMN NETosis and decreased phagocytosis in cattle (Ingvartsen and Moyes, 2015). In addition, immune activity is limited by low glucose availability, the primary energy source of the immune system (Ingvartsen and Moyes, 2015). The endocrine changes and nutrient shift may also contribute to the low energy state of the mammary gland during the peripartum period.

Immune cells of the mammary gland are affected by endocrine signals and paracrine signals. Increased estrogen promotes immunosuppression of regulatory T cell populations (Habib *et al*., 2018). Progesterone and prolactin, meanwhile, raise regulatory T cell populations indirectly during pregnancy and lactation, respectively (Leehy *et al*., 2018). Involution is another time of great mastitis incidence. This is especially true of active involution as the mammary gland continues to secrete milk that increases intramammary pressure on the teat meatus and sphincter muscles. The remodeling of the mammary gland during involution also occupies the resident immune cells with dead cell or milk debris in the alveolar lumen. Ultimately these factors result in decreased immune cell response to infection in the involuting mammary gland.

The immune status of the mammary gland is due in part to mucosal immunity. The apical membrane of the lactating mammary epithelium secretes an ECM and a mucus border that provides immune components such as IgA from CCL28-recruited B-cells and pore forming mucins (Wilson *et al*., 2004). The mucosal lining also acts as a barrier in addition to the ECM against the immune components and free radicals found in the milk (Ballard *et al*., 2013). The mucosal lining is also where antigen presenting dendritic cells reside. These cells are of interest because their ability to activate T-cells is suppressed in lactation and early involution but is most active by mid-involution (Betts *et*

al., 2018). This contributes as a factor of increased mastitis susceptibility at early lactation and early involution.

The pathogen invades the mammary gland through the streak canal and Furstenberg's rosette, thus entering the teat and gland cisterns. To get here, the pathogen must get by the keratin plug, which immobilizes many bacteria, and the sphincter muscles that keep the teat end closed. From here, bacteria invade the mammary gland, proliferate, and produce toxins and metabolites which activate several signaling pathways. LPS binds to the CD14/MD2/TLR4 co-receptor that stimulates cytokine release and reactive oxygen species (ROS) production from MECs, PMNs, macrophages and other immune cells, and activates soluble immune factors (He *et al*., 2015).

Initially, the bacteria are targeted by soluble immune factors, such as beta-defensin, LPS-binding protein, lysozyme, lactoferrin, immunoglobulins and the complement system. These are synthesized by MECs into the milk with beta defensin and lysozyme forming pores in bacterial membranes to induce lysis and lactoferrin sequestering iron, a vital and volatile nutrient, from bacteria to inhibit their growth (Günther *et al*., 2009). MECs also secrete xanthine oxidase on milk fat globule membranes and lactoperoxidase which destroys bacteria in the presence of thiocyanate and hydrogen peroxide. IL-1B, TNF-alpha, and IL-6 are among the cytokines that initiate this response, and it has been shown that wound response genes such as vascular endothelial growth factor (VEGF) are induced to support angiogenesis (Younis *et al*., 2016). Immune components such as the complement system, immunoglobulins, defensins and lysozymes are all upregulated at this initial stage while ECM genes are suppressed at this time (Younis *et al*., 2016). This is likely from increased extravasation and diapedesis

of PMNs from the bloodstream and into the mammary gland coinciding with a rise in milk sodium content and the loss of tight junction integrity. In fact, many of the mammary gland immune components diffuse from the serum, such as transferrin. Immunoglobulins (Ig), predominately IgG1 in bovine milk, opsonize pathogens and their products to render them inert and mark them for destruction by macrophages early on.

In severe cases of mastitis, the persistence of infection in the mammary gland stimulates the adaptive immune system as the cytokine signaling of the innate immune system reaches the supramammary lymph node, the crossroads of the innate and adaptive immune systems of the mammary gland. T-cell proliferation is stimulated, and B-cells differentiate into plasma cells that actively produce antibodies or become memory cells to prevent future infection. The increased permeability of the mammary epithelium and vascular endothelium allows for secreted toxins to enter the bloodstream and exert systemic effects. The action of TNF-α, IL-2, IL-6, and IL-8 stimulates the production of eicosanoids, leukotrienes and prostaglandins. The former aid in the recruitment of more PMNs and increase vascular permeability, while the latter promotes vasodilation and a systemic febrile response in the host.

Mastitis in *E. coli* infected cows decreases the relative expression of α-S1-casein 1.8-fold and β-casein 2.5-fold, and *E. coli* LPS challenge results in a 75% reduction in milk yield within 24 hours (McFadden *et al*., 1987). Intramammary challenge of cattle with *E. coli* LPS drastically changes the metabolism from a predominately oxidative one via mitochondrial processes to a primarily glycolytic metabolism in the cytosol (Silanikove *et al*., 2011). This change in metabolism lowers the energy state of the cell, thus the production of proteins and lactose decrease. This shifts the nutrients to help the

immune system and produce antibacterial metabolites lactate and malate in the milk by MECs (Silanikove *et al*., 2011). In accordance with this, Burgos *et al*. (2013) found that AMPK, which is activated by low energy signals, inhibits mTORC1 through TSC1-2 and decreased phosphorylation of 4E-BP1 and SK61.

1.4.4. Economic Impact to Dairy Industry

The dairy industry loses a great amount money to mastitis. Economic tolls attributed to mastitis are mostly associated with reduced milk production. Holland *et al.* (2015) devised a direct comparison interaction model and determined that milk yield losses due to mastitis were 0.0388 kg/day per 1% rise in somatic cell count. This model allows economic deficits to be related to mastitis severity by normalizing the cost in losses to the number of cells shed in the milk. In the United States alone, mastitis accounts for $2 billion in losses. Globally it costs an estimated $533 billion (Shaheen *et al*., 2016). While there are other costs such as veterinary care and pre-mature culling, mastitis inflicts its greatest impact through losses in milk production.

1.5. Oxidative Stress and Antioxidation

Oxidative stress occurs when the number of oxidants outnumbers the antioxidative capacity of a tissue. A contributing factor in a multitude of conditions in disease, oxidative stress arises from a multitude of sources and inflicts damage to the host directly and indirectly. To combat these assaults, the antioxidative response is stimulated through the forkhead box, class one (FOXO) and the kelch-like ECH-associated protein 1 (KEAP1) - nuclear erythroid 2-related factor 2 (Nrf2) signaling pathways (Klotz *et al*., 2017; Plafker *et al*., 2010).

1.5.1. Sources of Oxidative Stress

Superoxide is produced in the electron transport chain (ETC) of the mitochondria in addition to enzymatic reactions, such as reactions by xanthine oxidase and those seen in lysosomal degradation and peroxisome proliferation (Cerruti *et al*., 1985). The mitochondrion contains its own DNA to maintain itself, but it is subjected to oxidative damage when free radicals are produced from the ETC (Bhat *et al*., 2015). This damage exacerbates the ROS issue because the damaged DNA impairs the mitochondrion's synthesis of ETC components and antioxidative components, such as superoxide dismutase (SOD) (Bhat *et al*., 2015). Hydrogen peroxide, when provided iron in the case of cytochrome-c or another source of electrons, can produce hydroxyl radicals via the Fenton reaction with iron or form superoxide in the case of the Haber-Weiss reaction (Fenton, 1894; Haber and Weiss, 1934).

ROS can also be produced as a result of the immune system. Concentrated ROS are utilized in immune signaling and as a weapon by the cell against pathogens, which leads to targeted destruction with collateral damage. The activation of the immune system and inflammation stimulates inducible nitric oxide synthase to produce nitric oxide, which is a potent signaling molecule that stimulates vasodilation (Lind *et al*., 2017). Hydroxyl radicals are also produced by activated PMNs by the formation of hypochlorous acid and peroxynitrite by myeloperoxidase (Prutz, 1996). The production of free radicals from multiple sources adds up to a massive oxidative burden to the microenvironment, thus the immune response often results in oxidative stress.

While ROS cause damage, they are naturally produced in the cell and are an integral part of cellular signaling. Proteins, particularly cysteine and methionine

containing proteins, are capable of being temporarily modified by ROS. Cysteine and methionine contain sulfur groups that are easily oxidized to hydroxylated or nitrosylated sulfur groups by ROS and RNS, respectively, and can be reverted to their previous form by glutathione or other reducing equivalents (Filomeni *et al*., 2015). An example of this nature is exemplified by glutathione when it is oxidized by a free radical and the now oxidized glutathione dimerizes with another oxidized glutathione in oxidative conditions. Another example of this control is seen in apoptosis regulated signaling kinase 1 (ASK1), a regulator of JNK and p38. ASK1 under a balanced redox state is constantly inhibited by reduced glutathione, but it becomes more and more activated by ROS as glutathione is oxidized to combat the free radicals (Ray *et al*., 2012). While ROS are produced by mitochondrial distress and immune actions, they are integral signaling molecules as well.

1.5.2. Implications of Oxidative Stress

Oxidative stress and its effects are mediated by oxidants, which can be classified into 2 main classes: reactive oxygen species (ROS) and reactive nitrogen species (RNS). ROS and RNS are each derived from oxygen. Oxygen itself is considered a radical, an oxidant with an unpaired electron, along with superoxide, oxygen radical, hydroxyl, nitric oxide, and nitrogen dioxide. The other main class of oxidants are non-radical species, which are molecules prone to participate in free radical reactions, and some of these molecules include hydrogen peroxide, singlet oxygen, hypochlorous acid, and peroxynitrite. The nature of how these molecules promote oxidative stress differs from molecule to molecule.

Superoxide can oxidize iron complexes like cytochrome C and can produce perhydroxyl radical, which inflict lipid peroxidation (Cerruti *et al*., 1985). It is resolved

by superoxide dismutase, where it is converted into oxygen diatom and hydrogen peroxide (Bielski *et al*., 1996; Bielski *et al*., 1985). Hydrogen peroxide is eliminated by catalase and glutathione peroxidase (Halliwell *et al*., 2000). Hydroxyl radicals are very reactive and can inflict damage to carbohydrates, lipids, nucleic acids, and proteins. They are also produced by activated PMNs by formation of hypochlorous acid by myeloperoxidase (Prutz, 1996). Hypochlorous acid itself is involved in chlorination and oxidation reactions that form chloroamines from amines and chlorohydrins from cholesterol and unsaturated lipids (Prutz, 1996). PMNs can also form peroxynitrite, which can further breakdown into hydroxyl radical and nitric oxide. Nitric oxide is lipid soluble and is an integral signaling molecule that mediates vascular responses and monitors redox state via protein nitrosylation of certain molecules (Wink and Micthell, 1998; Stamler, 1994)

DNA is highly susceptible to oxidative stress, particularly mitochondrial DNA as it is in the environment of oxidative phosphorylation and increased metabolic activities that promote oxidative stress. Hydroxyl radicals among others can cause single and double stranded breaks in DNA and can abstract hydrogen to cause cross linking. Of all the byproducts created by this destruction, 8-hydroxy-deoxyguanosine is utilized as a marker of oxidative DNA damage (Barja *et al*., 2000). Peroxynitrite can perform both oxidative and nitrative DNA damage via the production of 8-deoxyoxyguanosine and 8-nitroguanosine, respectively (Hiraku, *et al*., 2010). In RNA, ROS attack is much more common due to several factors: RNA is single stranded and more prone to breaks; oxidized RNA cannot be reversed; there is little protective mechanism for RNA from ROS unlike DNA; and RNA exists in the cytosol where ROS are used in signaling and

generated as a byproduct from the mitochondria (Abe *et al*., 2002; Martinet *et al*., 2004). The primary marker of oxidative damage to RNA is 7,8-dihydro-8-oxoguanosine and is elevated in diseases like Alzheimer's disease and atherosclerosis (Abe *et al*., 2002; Martinet *et al*., 2004).

Lipids are susceptible to oxidation by free radicals, which is a process termed lipid peroxidation. Particularly, the unsaturated bonds of monounsaturated fats like alpha-linoleic acid and polyunsaturated fats such as arachidonic acid are reductive and attract electrons (Mavangira *et al*., 2018). A lipid is oxidized when a hydrogen is abstracted to form a lipid radical that reacts with diatomic oxygen to create a lipid peroxyl radical that catalyzes the formation of endoperoxides. This leads to the formation of lipid peroxidation's toxic products malondialdehyde and 4-hydroxyl noneal (Bast *et al*., 1993). Another class of oxidized lipids are isoprostanes, which are arachidonic acid derivatives following peroxidation and are markers of oxidative damage (Aruoma, 1998). The resulting impacts are not only damage to DNA and proteins, but also decreased membrane fluidity, integral protein function, and propagation of the oxidative reaction to other lipids.

Proteins are susceptible to all the ROS and RNS species listed previously (Dean *et al*., 1997). Amino acids, particularly cysteine and methionine, are oxidized to form cross-linkages that ultimately denature proteins and inhibit their function (Butterfield *et al*., 1998). Even though the sulfur-containing amino acids are more susceptible to oxidation, their oxidation is reversed by the action of disulfide reductases and methionine sulfoxide reductases. Markers of oxidative damage to proteins include 3-nitrotyrosine by RNS or

O-tyrosine by OH- in addition to protein carbonyls in the case of arginine, lysine, proline, and threonine (Chevion *et al*., 2000).

1.5.3. The Antioxidative Response

As stated earlier, oxidative stress is dependent on the balance between generation of molecules capable of redox reactions and pathways to minimize their generation. Any substance that delays or resolves oxidative damage to affected molecules is known as an antioxidant. Antioxidants in mammals can be defined as either enzymatic or non-enzymatic effectors. Of the enzymatic effectors, superoxide dismutase is the most well documented with others like glutathione peroxidase, thioredoxin reductase, and NADPH quinone reductase. These antioxidative enzymes are closely regulated by redox signaling pathways. Particularly, the FOXO and KEAP1-Nrf2 signaling pathways are the principal signaling pathways responsible for maintaining redox status.

FOXO is a class of transcription factors associated with the response to foreign compounds. FOXO is upregulated by adverse cellular stress, such as oxidative stress, and in a normal cell, FOXO is inhibited by PI3K/Akt signaling. Oxidative stress not only inhibits PI3K/Akt signaling activation, but also promotes binding of FOXO to its promoter, CREB-binding protein (CBP) and phosphorylation by JNK (Klotz *et al*., 2017; Carter *et al*., 2008). Activation of FOXO leads increased synthesis of factors involved in cell cycle arrest, cell death, DNA repair, glycolysis, and enzymes involved in antioxidation like catalase (Klotz *et al*., 2017).

Like FOXO, Nrf2 is also upregulated by oxidative stress as well as PI3K/Akt signaling. Nrf2 consists of 7 Nrf2-ECH domains (Neh1-7). Neh1 is a basic leucine zipper that binds musculoaponeurotic fibrosarcoma protein (Maf) and ubiquitin-conjugating

enzyme UbcM2 and binds to DNA (Plafker *et al*., 2010; Itoh *et al*., 1997). Nrf2 is constitutively expressed and is regulated in 2 different manners: KEAP1-dependent or KEAP1-independent. In the KEAP1-dependent mechanism, KEAP1 binds to motifs in Neh2 domain of Nrf2 and recruits cullin-based E3 ubiquitin ligase to target Nrf2 for polyubiquitination and proteasomal degradation (Hayes *et al*., 2010). In conditions of oxidative stress, the cysteine residues on KEAP1, which bind to Neh2, are oxidized and release Nrf2 (Hayes *et al*., 2010). KEAP1 may also be inhibited by p62 for lysosomal degradation to stabilize Nrf2 (Komatsu *et al*., 2010, Lau *et al*., 2010).

KEAP1-independent Nrf2 regulation is mediated by glycogen synthase kinase 3 (GSK3), which phosphorylates serine residues on Neh6 motifs (Jain *et al*., 2010). This primes beta-transducin repeat containing protein that recruits Skp1-Cul1-Rbx1/Roc1 ubiquitin ligase complex to target Nrf2 for proteasomal degradation (Copple *et al*., 2010). Once Nrf2 is stabilized, it translocates to the nucleus and binds with Maf at Neh1 while Neh4 and Neh5 bind to SRC-3 and cAMP-response-element-binding protein (Smith *et al*., 2016). This complex initiates the transcription of target genes that contain antioxidant response elements (AREs) in the promoter for amelioration of oxidative stress.

AREs are contained by a wide variety of genes that all play a role in antioxidation on multiple levels. The major levels of response consist of enzymatic and soluble factor expression to mitigate free radicals. Enzymatic breakdown of free radicals is performed by heme oxygenase I (Hox1), NADPH quinone oxidase I (Nqo1) and other enzymes that destroy free radicals, replenish antioxidants, and ameliorate damages incurred in the tissue. Hox1 and Nqo1 are responsible for detoxification by neutralizing highly reactive heme from damaged cells and quinone free radicals from oxidative phosphorylation,

respectively (Ryter *et al*., 2016; Ross *et al*., 2017). Thioredoxin reductase is another ARE-inducible gene that helps replenish reduced glutathione with the help of cysteine/cystine transporter xCt, which provides thioredoxin reductase's substrates for glutathione synthesis (Mandal *et al*., 2010). AREs help the Nrf2 response operate in a coordinated manner with expression of enzymes.

The second phase, meanwhile, indirectly controls free radicals by keeping cell antioxidant capacity up with the oxidative burden via production of glutathione, tocopherol, retinol and other soluble factors. While glutathione has been previously noted, there are a wide variety of non-enzymatic antioxidants that possess the ability to give or accept electrons. Tocopherols are the predominant antioxidant in cell membranes as they can prevent the propagation of lipid peroxidation cascades (Mavangira *et al*., 2018). Ascorbic acid also participates in antioxidation by acting directly on free radicals and recycling other antioxidants as well (Yang et al., 2014). Lipoic acid is also a capable antioxidant that chelates metal, sequestering it from pro-oxidants, as well as in recycling dihydrolipoic acid (Baumgartner *et al*., 2017).

1.5.4. Oxidative Stress in the Mammary Gland

While the production of ROS is a natural part of signaling pathways and metabolic activities, it can be thrown into oxidative stress with the reduction of antioxidants. The mammary gland is no exception to this pathophysiology as total antioxidant capacity is reduced in the mammary gland in diseases such as mastitis.

In humans and cows, it has been documented that the periparturient period reduces the antioxidative capability of the mammary gland due to negative energy balance, which leads to inefficient action by leukocytes that extends into early lactation

(Abdelli *et al*., 2017). Because of negative energy balance, milk yield does not reach its peak output until the late phase of early lactation (Zachut *et al*., 2016). High amounts of oxidation can induce the mammary gland metabolism to shunt glucose away from glycolysis. It was found in cattle that there is an inverse relationship between energy balance and glucose 6 phosphate dehydrogenase, a rate limiting enzyme of the pentose phosphate pathway, with early lactation being a time of great G6PDH activity (Zachut *et al*., 2016). This potentially points to glucose funneling towards NADPH production, which fuels enzymatic free radical quenching and antioxidant reduction (Zachut *et al*., 2016).

There is a strong correlation between mastitis and a reduction in its damage with supplementation of antioxidants, such as vitamin E and selenium (Allison and Laven, 2000; Smith *et al*., 1984; Smith *et al*., 1997). Supplementation of vitamin E and selenium to cows increased the bactericidal behavior of macrophages in the mammary gland and blood relative to untreated cattle (Grasso *et al*., 1990; Gyang *et al*., 1984; Hogan *et al*., 1993). This may be due to the reduced adhesion and diapedesis of PMNs to the endothelium in selenium treated cattle (Maddox *et al*., 1999). Serum vitamin A is reduced in cows with clinical mastitis, and beta-carotene supplementation ameliorated the impact of mastitis (Chew, 1995). In accordance with vitamin A, vitamin C concentration in plasma decreases in mastitis afflicted cattle (Weis and Hogan, 2007). Cows with subacute ruminal acidosis presented LPS in the bloodstream, which lowered the total antioxidation capacity and expression of SOD1, SOD2, and GPx1 in bovine mammary gland tissue (Memon *et al*., 2019). The presence and breakdown of polyunsaturated fatty acids conjugated linoleic acid and docosahexaenoic acid, which can participate in free radical

sequestration, in human MECs induces Nrf2 activation and upregulated NQO1 and HO-1 expression, respectively (Bang *et al*., 2017).

Antioxidants are just the soluble measures of free radical control. The KEAP1-Nrf2-ARE antioxidant response pathway is a key component of the free radical balance of the mammary gland. Key endocrine modulators in the lactating mammary gland have shown to modulate Nrf2 signaling. Estrogen receptor alpha binding domain has been shown to be essential in inhibition of NQO1 expression (Yao *et al*., 2010). Estrogen related receptor beta overexpression in COS-1 cells has shown to reduce TBHQ-induced Nrf2 activation (Zhou *et al*., 2007), and glucocorticoid receptors have been shown to be inhibitors via interactions with the Neh4/5 domain of Nrf2 (Ki *et al*., 2005).

Overall, oxidative stress plays a central role in both normal signaling and immune function while also being a key pathological characteristic in a plethora of diseases. The ultimate consequence of free radical imbalance, oxidative stress can inflict damage to proteins, fats, and nucleic acids, thus hindering cell function. The mammary gland has a lot of interplay with oxidative stress, and while there are many soluble factors that can impact the antioxidant response alongside Nrf2 signaling, mastitis tremendously increases the free radical burden on the tissue to induce cell damage, death and reduced milk production.

1.6. Hypoxia

Oxygen needs are well known for oxidative metabolism, but the demand for oxygen differs within each tissue depending on metabolic rate, tissue location, and other factors. When the amount of available oxygen decreases below normal limits in a tissue, the hypoxic response is initiated to adapt the cell and tissue to this condition. The oxygen

content of a tissue can vary from as high as 21% in the lung to as low as 1% in the retina, for example (Shao *et al*., 2014). When the oxygen tension of a tissue drops below its minimum threshold, it activates hypoxia-inducible factor 1 (HIF-1) in an oxygen dependent manner. HIF-1 is a heterodimeric transcription factor consisting of a regulatory α-subunit and a β-subunit and both are constitutively produced. The former is regulated in conjunction with oxygen tension as oxygen concentration controls the activities of prolyl hydroxylases I-III (PHD) (Greer *et al*., 2012). PHDs under normal oxygen conditions add hydroxyl groups to HIF-1α, which binds von Hippel Lindau protein to induce polyubiquitination of HIF-1α and its subsequent degradation by the proteasome (Greer *et al*., 2012). In hypoxic conditions, the PHDs are inhibited, thus the transcription factor can bind to HIF-1β and transcriptional enhancer p300 followed by translocation to the nucleus to act on hypoxic response elements (HREs) of genes. Some notable HRE contained genes include glucose transporters, glycolytic enzymes and lactate dehydrogenase for anaerobic metabolism and VEGF to encourage angiogenesis for more oxygen delivery (Shao *et al*., 2014; Laukka *et al*., 2016).

While oxygen concentration is widely known to control HIF-1α, the inflammatory response is also a potent activator. The inflammatory response activates hypoxic signaling by HIF-1α at the gene and protein levels in an oxygen-independent manner. Inflammatory signaling is regulated in part by NF-κβ transcriptional regulation, which happens to directly control the gene expression of HIF-1α (Ge *et al*., 2019). At the protein level, inflammation raises the oxidative stress of the affected tissue, and elevated ROS have been shown to be inhibitors of PHD-mediated HIF-1α repression (Fitzpatrick *et al*., 2018). Immune cells, which operate on a largely glycolytic metabolism, have high

expression of HIF-1α relative to other cells, and HIF-1α expression is stimulated by LPS treatment (Fitzpatrick *et al*., 2018; Ge *et al*., 2019).

Hypoxia signaling plays an important role in immune signaling, yet it has not been explored as it relates to mastitis. The mammary gland has presented evidence that it experiences increasing amounts of hypoxia from mid-pregnancy to early lactation in mice (Shao *et al*., 2014). There may be a potential role that hypoxic signaling serves in mammary development as HIF-1α expression varies throughout mammary development and oxygen consumption increases from mid-pregnancy to early lactation (Shao *et al*., 2014; Påhlman *et al*., 2015). While the change of the concentration of oxygen in the mammary gland throughout a lactation is studied, the role of oxygen tension in mastitis remains to be elucidated.

1.7. Summary and Study Aims

Mastitis costs the dairy industry $2 billion annually in the U.S.A. While it is known that the major portion of this cost is due to a loss of production, the underlying mechanisms are largely unknown. We hypothesized that mastitis induces localized hypoxia in the mammary gland caused by leukocyte recruitment, which reduces overall milk production along with the combined actions of elevating cell death and oxidative stress and inhibiting milk gene expression. The specific aims were to examine the effects of intramammary LPS challenge on cell apoptosis, oxidative stress, oxygen tension, and milk protein gene expression in the mammary gland using a mouse model.

Chapter 2: Bacterial Endotoxin Increases Oxidative Stress and Oxygen Tension while Reducing Milk Protein Gene Expression in the Mammary Gland

Alexander Jonathan Spitzer, Qing Tian, Ratan Choudhary, and Feng-Qi Zhao*

Department of Animal and Veterinary Sciences, University of Vermont, 570 Main Street, Burlington, VT 05452

Short title: Repressive Mechanisms of Milk Production by Mastitis

* Correspondence: fzhao@uvm.edu (F.-Q.Z.)

2.1. Abstract

Mastitis, the inflammation of the mammary gland by bacterial infection, is one of the costliest diseases to the dairy industry primarily due to a loss in milk production. The aim of this study was to investigate the mechanisms underlying reduced milk production during mastitis. We hypothesized that bacterial endotoxin induces cell apoptosis, oxidative stress and increases hypoxia while inhibiting milk gene expression in the mammary gland. To test this hypothesis, mice were bred to pregnancy, and 3 days post-partum the left and right sides of the 4th pair of mammary glands were alternately injected with either the endotoxin liposaccharide (LPS, *E. coli* 055:B5, 100 ul of 0.2 mg/ml) or sterile PBS through the teat meatus. At 10.5 and 22.5 h post-injection, pimonidazole HCl, a hypoxyprobe, was injected intraperitoneally. At 12 or 24 h after the LPS injection, the fourth glands were individually collected (n=8 pairs) and analyzed for hypoxia, gene expression and oxidative stress. LPS treatment induced mammary gland inflammation as shown by increases in inflammatory cytokine expression ($P < 0.001$) and neutrophil recruitment at 12 and 24 h. LPS promoted cell apoptosis in a transient manner; an abundance of cleaved caspase 3 was evident only at 12 h after LPS challenge ($P = 0.02$). Increased H_2O_2 content was seen at 12 h ($P < 0.001$) but decreased dramatically after 24 h of LPS treatment ($P < 0.001$). Total antioxidative capacity tended to decrease at both 12 and 24 h ($P = 0.067$ and 0.061, respectively). In agreement with these findings, LPS activated Nuclear factor erythroid 2-related factor 2 (Nrf2) antioxidative signaling in the mammary gland, demonstrated by increased expression of its target gene *Nqo1* at 12 h ($P = 0.05$) and *xCT* at 24 h ($P = 0.076$). Hypoxyprobe staining, indicative of hypoxia, was greater in the alveoli of PBS-treated glands than LPS-treated glands at both 12 and 24 h.

This suggests oxygen tension rises in response to LPS treatment. Conversely, milk expression genes, β-casein gene (*CSN2*) and α-lactalbumin (*LALBA*), were inhibited by LPS treatment across time. Expression of α-S1 casein (*CSN1S1*) mRNA increased with LPS treatment at 24 h, but protein expression was reduced at this same time point ($P < 0.05$). In summary, intramammary LPS challenge incurs inflammation, augments cell apoptosis, induces oxidative stress and activation of the Nrf2 antioxidation pathway, increases oxygen tension, and inhibits milk protein expression in the mammary gland. This study provides functional insight into mechanisms of reduced milk production during mastitis and provides possible approaches to combat reduction in milk production, such as enhancing the Nrf2-antioxidative signaling pathway and reducing inhibition of milk protein expression.

Keywords:

Antioxidation, Gene expression, Hypoxia, Inflammation, Mastitis, Nrf2

2.2. Introduction

Mastitis is a major economic challenge to the dairy industry. Mastitis is inflammation of the mammary gland caused by bacterial invasion and infection. The major reason for the economic loss is reduced milk production (Janzen *et al*., 1970; Guimarães *et al*., 2017). Mastitis is characterized by many forms, from acute clinical mastitis to chronic subclinical mastitis, as it is caused by a plethora of bacteria. Clinical mastitis causes the mammary gland to become swollen and inflamed by vasodilation, and it is often painful for the mother. In contrast, subclinical mastitis is often asymptomatic, but still reduces milk yield. During mastitis, immune cell recruitment to the site of infection enhances somatic cell count (SCC). Additionally, compromised mammary epithelial cell (MEC) tight junctions permit increased sodium content in the milk (Leitner *et al*., 2004). Together, SCC and sodium content in the mammary gland increase with severity of mastitis, and milk yield decreases. Mastitis not only causes acute effects to the cow, but it also leads to chronic issues. For example, milk production in animals with resolved mastitis is much lower than unafflicted animals (Boscos *et al*., 1996; Dürr *et al*., 2008, Wilson *et al*., 1995). In subclinical mastitis, the development of antibiotic resistance adds costs to veterinary care and can result in premature culling (Vanderhaeghen *et al*., 2010; Aslantaş *et al*., 2016; Hinthong *et al*., 2017).

The type of infection in mastitis is in part dependent upon the etiological agent. Subclinical mastitis is most often caused by Gram-positive bacteria, whereas Gram-negative bacteria commonly cause acute clinical mastitis. Gram-positive bacteria that cause mastitis include *Staphylococcus aureus (S. aureus)*, *Streptococcus agalactiae (S. agalactiae)* and *Streptococcus uberis (S. uberis).* These pathogens spread mostly by

physical contact between animals and people, which is why it is considered contagious mastitis. Gram negative bacteria, on the other hand, are usually spread through exposure to fecal matter. These bacteria are often pathogenic strains of *Enterobacteriaceae* found in mammalian gastrointestinal tracts, such as *Escherichia coli (E. coli).* These bacteria are not as adept at evading the host immune system, and thus they are readily targeted for destruction. The lysis of these bacteria by soluble factors like β-defensin triggers the release of endotoxins such as lipopolysaccharide (LPS), a superantigen that overstimulates the host immune system to cause acute inflammation, endotoxemic shock and other symptoms of clinical mastitis (Bhattarai *et al*., 2018; Hagiwara *et al*., 2016). Interestingly, heat inactivated *E. coli* activate pro-inflammatory signaling more strongly than heat inactivated *S. aureus* (Bhattarai *et al*., 2018).

Bacterial infection in tissues initiates a cascade of innate immune responses. Macrophages and MECs work in concert to recruit polymorphonuclear neutrophils (PMNs), which in turn combat pathogens via phagocytosis and the expression of reactive oxygen species. PMNs also secrete cytokines that support a pro-inflammatory and antimicrobial environment. However, resolving the infection is not without costs. Tight junctions of MECs are compromised by pro-inflammatory responses and PMN diapedesis (Wang *et al*., 2017, Elazar *et al*., 2010). Other MECs undergo apoptosis (Xiao *et al*., 2015; Nagasawa *et al*., 2017; Matsunaga *et al*., 2018). Because of the dramatic increase in PMNs recruited to the infected areas, we hypothesized that mastitis may cause localized hypoxia due to increased oxygen consumption by the PMNs.

Massive PMN recruitment also increases free radical production to unbalanced levels, a pathological state known as oxidative stress. Bacterial toxins and the immune

response work in concert to raise oxidative stress during infections, which stimulates an antioxidative response. Soluble factors, such as glutathione and vitamin E, help to immediately resolve oxidative stress, but the efficacy of these factors diminish as oxidative stress increases (Mir *et al*., 2017; Aref *et al*., 2018). To restore the balance, the nuclear erythroid 2-related factor 2 (Nrf2) - antioxidant responsive element (ARE) pathway is activated, which also stimulates the synthesis of antioxidant enzymes, such as heme oxygenase-1 (Hox1) and NAD(P)H quinone oxidoreductase 1 (Nqo1) (Wang *et al*., 2017). Together, these factors reduce the oxidant content and reestablish redox balance.

Because of its impact to dairy industry, mastitis research has become an increasingly progressive field as many look to the pathology of this disease and finding therapeutic treatments to combat it. The most common approach to study mastitis has been through an intramammary infusion of pro-inflammatory bacteria toxin using bovine and rodent models (Hoeben *et al*., 2000; Jiang *et al*., 2018). Many studies utilize vitamins, minerals, or known antioxidants to investigate their therapeutic potential to prevent damage incurred by mastitis in cattle (Minuti *et al*., 2015) and in mice (Wang *et al*., 2017). While they do not fully mimic all aspects of bovine lactation, mice and rats are widely used as mastitis models because they are easier to maintain and they are cost effective (Chandler, 1970). Importantly, the findings from rodent models of mastitis have largely confirmed bovine phenomena (Chandler, 1970; Lee *et al*., 2003; Notebaert *et al*., 2006; Breyne *et al*., 2015; Gogoi-Tiwari *et al*., 2017). Whereas most of these studies have examined effects of potential therapeutics on inflammation, oxidative stress, and apoptosis in the mammary gland, studies investigating the effects of bacterial toxins on

milk protein expression are limited, and no study to our knowledge has examined oxygen tension in the mammary gland during mastitis.

The objective of this study was to investigate the time-dependent effects of bacterial LPS challenge on oxygen tension, oxidative stress, and expression of milk protein genes and genes involved in these pathways in the mammary gland using a mouse model. Our study showed that LPS incurs inflammation, augments cell apoptosis, induces oxidative stress and activation of the Nrf2-ARE antioxidation pathway, increases oxygen tension, and inhibits milk protein expression in the mammary gland. All of these effects likely contribute to the decrease in milk production during mastitis.

2.3. Materials and Methods

2.3.1. Animal Treatment and Tissue Isolation

Sixteen 8-week-old female BALB/cJ mice (Jackson Labs, Bar Harbour, ME) were used for this study, and all procedures of animal use were approved by the University of Vermont Institutional Animal Care and Use Committee (IACUC Protocol #17-030). Mice were kept in breeding cages in harems of 1 male and 2 females under a controlled environment (25˚C, 45% humidity and 12 h light-dark cycle) and were bred to pregnancy. Three days postpartum, all lactating mice were anesthetized with 4.0% isoflurane and then received an intramammary infusion (IMI) of either lipopolysaccharide (LPS, *E. coli* 055:B5, #6529, Sigma, St. Louis, MO; 100 µL of 0.2 mg/mL) or sterile PBS (100 µL), alternatively into either side of the 4th mammary glands through the teat meatus with 30G 0.5 mL insulin syringes (Becton, Dickinson & Company, Franklin Lakes, NJ), and the injection site was thoroughly and gently massaged. At 10.5 and 22.5 h post-IMI, 1.5 mg/mL pimonidazole HCl (#HP2-1000 kit,

Hypoxyprobe, Burlington, MA) in sterile water was injected intraperitoneally (*i.p.*) into each mouse at 4 µL per gram body weight. At 12 or 24 h after the LPS infusion, eight mice were euthanized by cervical dislocation to maintain oxygen tension in the microenvironments of the tissues. The 4th glands were individually collected immediately from all animals (n=8), and tissue samples were either snap-frozen in liquid nitrogen and later stored in -80ºC or chemically fixed as described below.

2.3.2. RNA Isolation, Reverse Transcription (RT) and Real-Time Quantitative Polymerase Chain Reaction (qPCR)

Frozen mammary tissue was weighed to 30 mg, dispersed and ground by mortar and pestle chilled in liquid nitrogen. Ground tissue was placed directly into 600 µL of lysis buffer (RLT, Qiagen, Venlo, Netherlands) with 1% β-mercaptoethanol (#M6250-100 mL, Sigma) and was homogenized by an electric homogenizer in 3 x 10 s bursts with 10 s in ice between bursts to avoid rise in temperature. Total RNA was then isolated using RNEasy Mini Kit (#74104, Qiagen) according to the manufacturer's instruction. Total RNA was treated with DNAse I (#79254, Qiagen) and eluted in ultrapure water. The RNA concentration and quality were evaluated by Nanodrop 2000 spectrophotometric analyzer (#ND-2000, Thermo-fisher, Waltham, MA), and RNA quality was further analyzed by Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA) at the Vermont Integrated Genomics Core (Burlington, VT) to verify RNA Integrity Number values ≥ 8.0.

cDNA synthesis was performed using Invitrogen SuperScript III cDNA Synthesis Kit (#18080051, Invitrogen, Waltham, MA) according to manufacturer's specifications in an Applied Biosystems 2720 Thermocycler (#4359659; Applied Biosciences, Waltham,

MA). RNA samples were diluted to 2 µg for 8 µL reaction with ultrapure grade water with 1 µL each of provided dNTP mix and Oligo(dt-20). Samples were denatured at 85ºC for 5 min and then added with 10 µL of SuperScript III reaction mix. Reactions were performed at 50ºC for 50 min, 85ºC for 5 min and at 4ºC for 5 min. Reactions were collected by brief centrifugation for 1 min at 18,000 x g and 1 µL RNAse H was added to each reaction and incubated at 37ºC for 20 min before stored at -20ºC.

mRNA expression was determined by qPCR using iTaq SYBR Green Supermix (#1725121, Bio-Rad, Hercules, CA). 1 µL of cDNA solution was mixed with 500 nM forward and reverse primers (Table 1) in 10 µL, and 10 µL of iTaq SYBR Green Supermix was added to each reaction. The reactions were carried out in Bio-Rad CFX96 Thermocycler using the following program: initial denaturation at 95ºC for 30 s and 40 cycles of 5 s at 95ºC for denaturation and 30 s at 60ºC for annealing and extension followed by melt curve analysis from 65-95ºC at 0.5ºC increments at every 5 s. The qPCR data was analyzed by 2^^ (-ddCt) method (Livak, 2001) and normalized by the expression levels of the housekeeping genes *ActB*, *Hprt*, *Gapdh*, *Stx5a*, and *hnRnpab*. Specificity of primers was further assessed by the Vermont Integrated Genomics Core.

2.3.3. Protein Isolation and Western Blot

200 mg of frozen mouse mammary tissue was lysed in 1,500 µL NP-40 lysis buffer (#FNN0021, Fisher, Waltham, MA) with protease inhibitor cocktail (#8340, Sigma) and pulverized by Dounce homogenizer at 4ºC. Whole tissue lysates were then agitated at 4ºC for 2 h and centrifuged at 13,400 x g for 20 min at 4ºC. Supernatants were divided into aliquots and stored at -80ºC. Concentration of tissue lysates was determined by the Bradford Colorimetric assay (#5000001, Bio-Rad) with bovine serum albumin

(BSA) protein standard (#500-0007, Bio-Rad). Based on these concentrations, tissue lysates were diluted in lysis buffer to achieve a volume of 300 µL before addition of 100 µL 4X Laemmli buffer [62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% sodium dodecyl sulfate (SDS), 0.00125% (weight/volume) bromophenol blue] for a final protein concentration of 3.33 µg/µL. Samples were vortexed and immediately placed in 100ºC heat block for 10 min.

15 µL samples were loaded into gels for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Stacking gel: 0.125 M Tris-HCl, pH 6.8, 0.05% ammonium persulfate, 0.1% TEMED, and 4% SDS-polyacrylamide; Separating gel: 1.5 M Tris-HCl, pH 8.3, 0.05% ammonium persulfate, 0.05% TEMED, and 12% SDS-polyacrylamide) and ran at 150 V in electrode buffer (1.5% Tris base, pH 8.3, 7.2% glycine, and 0.5% SDS) until the dye front reached the end of the gel. The separated proteins were transferred to polyvinylidene fluoride membranes at 95 V and 4ºC for 90 min with transfer buffer (25 mM Tris, pH 8.3, 192 mM glycine, and 20% methanol), and the blots were stained with 1% ponceau S solution to evaluate protein transfer efficiency. Blots were blocked in 5% BSA in Tris-buffered saline with 0.1% Tween-20 (TBS-T, #P1379, Sigma).

Blots were incubated overnight on a plate shaker at 4ºC with primary antibody for β-Casein (#sc-166684, Santa Cruz Biotechnology, Santa Cruz, CA; 0.2 ug/mL), α-S1-Casein (#sc-365929, Santa Cruz Biotechnology; 0.2 ug/mL), α-Lactalbumin (#abx101423, Abbexa, Cambridge Science Park, Cambridge, United Kingdom; 0.2 ug/mL), and Cleaved Caspase 3 (#CST-9661, Cell Signaling, Danvers, MA; 1:1000). Blots were washed 3 times with TBS-T for 10 min intervals followed by incubation with secondary antibodies [goat horseradish peroxidase (HRP)-linked anti-rabbit IgG: #CST-

7074P2, Cell Signaling; Goat HRP-linked anti-mouse IgG: #sc-2005, Santa Cruz Biotechnology] at 1:10,000 dilution and room temperature for 1 h (Santa Cruz Biotechnology). Blots were then washed 4 times in TBS in 10 min intervals before incubation with ECL-substrate solutions (#34577, Invitrogen). Blots were then imaged with ChemiDoc ECL Imager (Bio-Rad), stripped for 15 min in Restore western blot stripping buffer (#21063, Invitrogen), and reprobed with antibody for GAPDH (#2118, Cell Signaling; 1:5000). Optical density of images was evaluated by ImageJ (Java-based image-processing and analysis software, ver. 5.12a; National Institute of Health, Bethesda, MD; Gallo-Oller *et al.*, 2018) and normalized by the expression of GAPDH.

2.3.4. Histological Staining and Immunohistochemistry

Fresh mouse mammary tissue was cut into 3-5 mm pieces and either fixed in 4% paraformaldehyde and embedded in optimal cutting temperature (OCT) compound or fixed in 10% formalin and embedded in paraffin. For OCT embedding, fresh tissues were washed 3 times in ice cold sterile PBS and immersed in PBS with 4% paraformaldehyde at 4ºC for 4 h. After 3 washes in ice cold PBS, the tissue pieces were immersed in 0.5 M sucrose in PBS at 4ºC overnight, embedded in OCT compound, and then stored at -80ºC. For paraffin embedding, tissue pieces were fixed in neutral buffered PBS with 10% formalin (#HT501128, Sigma) at room temperature for 4-6 h and stored in 70% ethanol at room temperature. Tissue embedding, deparaffinization, sectioning, hematoxylin and eosin staining were performed by the Pathology Department of the University of Vermont Medical Center (Burlington, VT).

Immunostaining of mammary tissue was performed as per the literature (Choudhary *et al.*, 2018) with modifications. Briefly, 5 µm thick tissue sections were

deparaffinized in xylene (3 x 5 min) followed by dehydration in absolute ethanol (2 x 3 min), gradual rehydration of tissue sections in 95% ethanol (2 x 3 min), 70% ethanol and deionized water (2 x 2 min). Antigen retrieval was done on hot plate for 10 min in boiling citrate buffer at pH 6.0 (Vector lab, Burlingame, CA) followed by 30 min of cooling at room temperature. Slides were washed in PBST (PBS with 0.5% of tween 20). After blocking non-specific protein binding with 2.5% horse serum (Vector lab) for 20 min, slides were incubated with FITC conjugated anti-pimonidazole mouse IgG1 monoclonal antibody (1:100 dilution in 2.5% horse serum; Hypoxyprobe™ Plus Kit, Hypoxyprobe, Inc., Burlington, MA) for 1.5 h at room temperature in moist chamber. Slides were washed with PBST (3 x 2 min) before mounting with Vectashield with 4,6-diamidino-2-phenyl-indole (DAPI) (Vector Lab). Slides were viewed with a fluorescence microscope (model Eclipse 50xi, Nikon Instruments Inc. NY,) at 200X magnification under green (FITC) and blue (DAPI) channels.

Five to seven images/slide were captured from LPS- and PBS-treated glands under constant illumination. Images were opened in ImageJ (ver. 5.12a), and inner and outer boundaries of alveoli were marked using freeform drawing tool. From each image, 12 distinct and intact alveoli were marked and measured for AREA, INTEGRATED DENSITY, STANDARD DEVIATION and MEAN GRAY VALUE of hypoxia signal using freeform drawing tool. The difference in raw integrated intensity of outer and inner boundary of alveoli provided the mean integrated intensity of hypoxia probe signal of mammary epithelium.

2.3.5. Oxidative Stress and Antioxidation Assays

Hydrogen peroxide (H_2O_2) levels in mammary tissues were analyzed using Sigma Fluorimetric Hydrogen Peroxide Assay Kit (#MAK166) according to manufacturer's specifications. Briefly, protein lysates from Western blot analysis were diluted to 1 mg/mL in NP-40 lysis buffer without protease inhibitors to avoid downstream interference of the assay. In 96 well plates, 50 µL of each samples and standards of 0, 0.1, 0.3, 1, 3, and 10 uM H_2O_2 was laid out and each reaction was mixed with 50 µL reaction mixture consisting of assay buffer with 20 units/mL horseradish peroxidase and 1% infrared fluorometric peroxidase substrate. Plates were incubated at room temperature (23ºC) for 10 min and then read fluorometrically at 640 nm excitation/680 nm emission on a H4 Plate Reader (Biotek Synergy, Winooski, VT). Fluorescence of standards were utilized to form a standard curve and average fluorescence of samples and their dilution factor were used to quantitate the amount of H_2O_2.

Mammary gland total antioxidant capacity was analyzed using Sigma Antioxidant Assay Kit (#CS0790) according to manufacturer's specifications. Approximately 100 mg tissue was lysed in Dounce homogenizer in Assay Buffer at 4ºC. Lysates were centrifuged at 12,800 x g at 4ºC for 15 min, and the supernatants were collected and aliquoted. For each reaction, 10 µL 10 µg/mL tissue lysate was mixed with 10 µL horse myoglobin in ultrapure water. Standard curve was prepared with (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) at 0, 0.015, 0.045, 0.105, 0.21, and 0.42 mM, respectively. Before adding reaction buffer, 25 uL 3% H_2O_2 was mixed with 10 mL ABTS buffer to activate the reagent. To each reaction in a 96 well plate, 150 µL ABTS reaction buffer with H_2O_2 was added to reactions and incubated for 5 min at room

temperature (23ºC). 100 µL stop buffer was added, and plate was read for optical density at 405 nm in a Biotek Synergy 2 Plate Reader. The total antioxidant capacity of tissue samples was derived from the standard curve derived from the absorbance of the standards and the dilution factor of the sample.

2.3.6. Statistical Analysis

All the data were analyzed by two-way mixed ANOVA using PROC MIXED model in SAS (ver. 9.4; SAS Institute, Cary, NC). Time (12 and 24 h) and treatments (LPS and PBS) and side of treatment (Left or Right) fixed factors each at two levels. Animal and time were not repeated as each animal was euthanized for tissue collection, and different groups of animals were used at two time points. The effects of intramammary treatments (LPS and PBS) were observed over time, thus treatment was considered a repeated measure. Observations for each variable were the dependent variables in the model. Effects of animals were considered as random effects. Differences in means of each variable were detected by type 3 tests for fixed effects (treatment and time) and interactions (time*treatment). Data are expressed as mean ± SEM, and the differences between means were declared significance ($P < 0.05$) or with a tendency of significance ($0.05 < P < 0.10$).

2.4. Results

2.4.1. LPS Incurs Inflammation

Hematoxylin and eosin staining of tissue sections showed that at 12 h of treatment, intramammary challenge of LPS induced massive recruitment of cells, likely PMN based on cell morphology (Elazar *et al*., 2010), into alveoli compared to PBS challenge (Figures 1A and 1B), and this recruitment was also evident at 24 h of LPS treatment (Figures 1C and 1D). Moreover, mRNA expression of inflammatory cytokines IL-1B, IL-6, and TNF-

α was dramatically induced in the mammary gland by LPS at both 12 and 24 h ($P < 0.05$), but no difference was seen across time (Figures 1E-1G & Table 2).

2.4.2. LPS Induces Mammary Apoptosis

The effect of LPS on mammary gland apoptosis was investigated by the analyses of proapoptotic marker expression. Compared to PBS treatment, LPS did not affect mRNA expression of either caspase 3 or caspase 9 at 12 h and 24 h (Figures 2A & 2D, Table 3), but expression of cleavage-activated caspase 3 (CC3) protein was significantly increased (1.33-fold) within 12 h ($P = 0.02$, Figures 2B & 2C). LPS also increased mRNA expression of Bax by 4.3-fold at 12 h ($P = 0.001$), but had no effect at 24 h (Figure 2E, Table 3). Expression of the endoplasmic reticulum (ER) stress marker, Chop, was not affected by LPS at 12 h, but decreased dramatically at 24 h ($P = 0.006$, Figure 2F). Expression of *Bax* and *Chop* exhibited a time and treatment interaction ($P < 0.05$, Tables 2 & 3). Surprisingly, the mRNA expression of caspases 3 and 9 and Bax showed a side difference of the glands and particularly, expression in the left side presented an interaction with treatment in caspase 9 ($P < 0.05$, Table 3). No significant differences were observed in the expression of *Bcl2*, *CytS*, and *Bid* mRNA between the two treatments at any time (Figures 2G-2I & Table 2).

2.4.3. LPS Elevates Oxidative Stress and Stimulates the Antioxidative Response

LPS treatment induced oxidative stress in the mammary gland at 12 h, demonstrated by an increase (1.8-fold) in tissue H_2O_2 levels compared to PBS treatment ($P < 0.001$, Figure 3A). This effect diminished, however, by 24 h ($P < 0.001$, Figure 3). Total antioxidative capacity (TAC) of the mammary gland tended to be lower at both 12

(P = 0.066) and 24 h (P = 0.061) following LPS treatment (Figure 3B & Table 2). These dynamics also demonstrated a time and treatment-dependent interaction (P < 0.05, Table 2).

LPS did not affect expression of Nrf2 mRNA, a master regulator of the antioxidative response in the mammary gland, at either 12 or 24 h (Figure 3C & Table 2). However, it did increase expression of the Nrf2 target gene, *Nqo1*, at 12 h (P = 0.050, Figure 3D), and tended to increase expression of cystine transporter, *xCt*, at 24 h (P = 0.076, Figure 3E). In contrast, LPS tended to decrease expression of *Hox1* mRNA at 24 h (P = 0.056, Figure 3F). There was time by treatment interaction in terms of *Nqo1* and *Hox1* mRNA expression, and *Hox1* expression differed between the two sides of the mammary tissue (P < 0.05, Tables 2 & 3).

2.4.4. LPS Increases Oxygen Tension in the Mammary Gland

To investigate whether LPS induces a hypoxic condition in the mammary gland, we injected LPS-treated mice with the hypoxyprobe pimonidazole HCl, which binds to amino acids and proteins under hypoxic conditions. To our surprise, fluorescent staining of pimonidazole adducts showed that LPS did not induce hypoxic conditions in the mammary gland at either 12 or 24 h (P < 0.05) (Figures 5A-D & Table 2). Expression of *Hif1a* and *Slc2a1* mRNA (hypoxia-induced genes) were not affected by LPS infusion, although *Slc2a1* expression increased at 24 h compared to 12 h (Figures 5E-F).

2.4.5. LPS Reduces Milk Protein Gene Expression

Compared to PBS-treated glands, mRNA expression of milk protein genes *Csn2* and *Lalba* in LPS-treated glands was decreased at both 12 h (68% and 59% of control,

respectively; $P < 0.05$) and 24 h (39% and 47% of control, respectively; $P < 0.05$) (Figures 4A & 4B). In contrast, mRNA expression of *Csn1s1* was not affected by LPS at 12 h but increased by 24 h ($P = 0.013$) (Figure 4C, Table 2).

At the protein level, expression of all three major milk proteins was inhibited by LPS at 12 and 24 h ($P < 0.05$), except there was no effect on Csn1s1 at 12 h (Figures 4D-G; Tables 2 and 3). A time by treatment effect of LPS on Csn1s1 protein expression, however, was evident ($P < 0.05$, Table 2). Lalba protein expression differed between the two sides of the mammary gland ($P = 0.009$; Table 3).

2.5. Discussion

The overall objective of this study was to investigate how mastitis causes a reduction in milk production. To approach this goal, we studied the time-dependent effects of the major bacterial endotoxin LPS on inflammation, apoptosis, oxidative stress, oxygen tension, and milk protein gene expression in the mammary gland using a mouse model. In this model, the fourth pair of mammary glands were unilaterally challenged with either LPS or PBS through the teats. The major advantage of this model is the higher power in statistical analysis due to the use of both treatment and control in the same animal (McFadden *et al*., 1987); whereas a potential weakness is the possibility of systemic effects of LPS. Although we cannot rule out the possibility that LPS injected to one gland might enter the blood stream and travel to the opposite gland, effects were minimal because cytokine expression and PMN recruitment remained low in the PBS-treated gland. Interestingly, there was a location effect in terms of caspases 3 and 9, and Bax. This effect has not been reported previously, to our knowledge, and its nature is not

known. Nevertheless, for most of our analyses, relative location of LPS infusion did not have an effect.

We utilized LPS as a pro-inflammatory endotoxin produced by the common mastitis pathogen *E. coli* as it has been commonly used in a plethora of intramammary infusion studies. The inflammatory responses induced by LPS have shown no difference from live bacteria inoculations in both bovine and murine models (Hoeben *et al*., 2000; Breyne *et al*., 2015). We used smooth LPS (sLPS), rather than rough LPS (rLPS), in this study because the common gram-negative causative agents of mastitis produce smooth LPS (Hinthong *et al*., 2017; Jiang *et al*., 2018). Some studies have compared the effects of each type of LPS on MEC *in vitro* and *in vivo* and found that rLPS greatly induced inflammation *in vitro*, but sLPS produced a pronounced, sustained response in bovine mammary gland *in vivo* (Védrine *et al*., 2018). This is potentially due to more CD14 targeting the larger O-antigen of sLPS to promote TLR4 signaling. Indeed, previous studies have also indicated that sLPS challenge greatly induces CD14 expression in MECs in a dose dependent manner (Zheng *et al*., 2006).

As expected, intramammary LPS challenge in this study incurred mammary inflammation, demonstrated by a large increase of PMN infiltration into the alveolar lumen. Recruitment of PMN by LPS has been well shown in bovine and mouse mammary glands in previous studies (Zheng *et al*., 2006; Elazar *et al*., 2010). This recruitment results in a large increase in somatic cell count (SCC) in milk and is induced by inflammatory mediators, including cytokines IL-1B, IL-6 and TNF-a, from macrophages and MECs (Elazar *et al*., 2010). The surge of expression of cytokines in the mammary gland by LPS challenge is also confirmed in this study.

In this study, mammary infusion of LPS significantly induced protein abundance of CC3 at 12 h, but not at 24 h, indicating a transient induction in cell apoptosis. Caspase 3 is a key death protease which plays a central role in the execution-phase of cell apoptosis by catalyzing the specific cleavage of many key cellular proteins (Yang *et al*., 2016; Fan *et al*., 2016). This enzyme is activated in the apoptotic cells both by death ligand-mediated and mitochondria-mediated pathways, and the activation of this enzyme requires proteolytic processing at conserved aspartic residues to produce two subunits (Yang *et al*., 2016). The cleaved active form of caspase 3 is a widely used marker of cell apoptosis. In supporting caspase 3 activation, mRNA expression of another popular cell apoptosis marker gene *Bax* showed the similar changes by LPS in this study. Bax forms a heterodimer with Bcl2 to activate cell apoptosis (Santos *et al*., 2018). These observations were consistent with the observations from a previous study in which intramammary infusion of LPS increased Bax expression at the mRNA and protein levels and caspase 3 cleavage at 12 h, but not caspase 3 gene expression (Song *et al*., 2014). Induction of cell apoptosis by LPS were also observed by TUNEL assay in mouse and bovine mammary tissue treated with LPS (Gao *et al*., 2016; Magro *et al*., 2017).

In this study, mRNA expression of an ER stress marker, Chop, is not increased at 12 h, but is decreased at 24 h following LPS challenge. Chop is a primary signaling proteins behind ER-stress induced apoptosis when the unfolded protein response is unable to resolve the source of stress (Oyadomari *et al*., 2003). The Chop depression at 24 h may suggest a dramatic improvement of anti-ER stress responses or a decrease of ER stress after 24 h. This observation also supports a possible decrease in cell apoptosis at 24 h in LPS infused glands.

Consistent with CC3 expression, LPS induced a temporary increase in oxidative stress in the mammary gland at 12 h, demonstrated by increased H_2O_2 level. Hydrogen peroxide is a natural metabolite and a signaling molecule in many organisms, but its overproduction by inflammatory and vascular cells during pathological conditions or over accumulation due to decreased antioxidation capacity can induce oxidative stress (Jia *et al*., 2019; Biswal *et al*., 2019). Milk from cattle with subclinical mastitis contains pronounced H_2O_2 content (Atakisi *et al*., 2010). Hydrogen peroxide is the product of superoxide and hydroxyl radical breakdown by enzymes like Sod1 and Nqo1, whose activities are increased by circulating LPS (Memon *et al*., 2019; Han *et al*., 2018). The accumulation of ROS can have destructive effects on cellular proteins, carbohydrates, lipids, and DNA to impair their functions and initiate apoptosis. In fact, it is widely documented that H_2O_2 can stimulate cell death in vitro by activating p53/Bax/Caspase 3 proapoptotic pathways in bovine MECs (Jin *et al*., 2015; Li *et al*., 2018).

However, the H_2O_2 level was largely decreased at 24 h of LPS treatment, suggesting a release and improvement of oxidative stress. This decrease was likely due to decreased production of ROS at this time because the TAC in the mammary gland tended to be decreased at both times after LPS challenge. This is also supported by our histological analysis which indicated reduced lymphatic cell infiltration, a major source of H_2O_2, into the mammary gland and is consistent with the *Chop* expression. The reduction of TAC by LPS or mastitis has been shown in previous studies. For example, cattle with subclinical mastitis showed lowered TAC with increased SCC in milk (Atakisi *et al*., 2010). Even increased LPS content in the plasma significantly lowered the TAC of

mammary tissue of cattle experiencing subacute ruminal acidosis incurred by a high-concentrate diet (Memon *et al*., 2019).

Nrf2 is a key regulator of antioxidation. During oxidative stress, it is activated by releasing from its binding with KEAP1 and then entering the nucleus to bind to the ARE in the upstream promoter of many antioxidative genes, including *Nqo1, xCt, Hox1, and Txnrd1* (Nguyen *et al*., 2003; Wible *et al*., 2018). To study its potential role in mastitis, we analyzed its expression as well as expression of its several target genes in this study. LPS did not change Nrf2 mRNA levels but upregulated the expression of Nqo1 at 12 h and tended to increase the expression of xCT at 24 h. Nqo1 and Hox1 are enzymes which breakdown ROS into water and oxygen (Agarwal *et al*., 2001; Ross *et al*., 2000). Our observations of gene expression of *Nqo1* and *Hox1* were consistent with the observation in LPS challenged bovine MECs in which *Nqo1* expression was greatest induced at 12 h, whereas *Hox1* was induced at only 4 h after the LPS challenge (Jin *et al*., 2015). The cysteine/cystine transporter xCt is required for the synthesis of the antioxidant glutathione and is Nrf2- and NF-kB-inducible with the latter being activated by ROS-induced ERK signaling (Lewerenz *et al*., 2012). Expression of *xCt* was greatly induced by LPS in primary bovine MEC cultures at 12 h, but less so at 24 h (Jin *et al*., 2015). It was shown that xCt activates MAPK following cystine supplementation to reduce effects of LPS-stimulated sepsis in mice via MAPK-dependent IL-10 anti-inflammatory signaling (Tanaka *et al*., 2015). Taken together, our observations indicated an activation of Nrf2-ARE pathway by LPS.

We originally hypothesized that the large recruitment of PMN to the mammary gland during mastitis could result in a local hypoxia. To test this hypothesis, we injected

LPS treated mice with the hypoxyprobe pimonidazole HCl which binds to amino acids and proteins in hypoxic regions. To our surprise, histochemical staining using FITC conjugated specific antibody to pimonidazole adducts in hypoxic cells showed that the MECs in LPS treated gland even had a higher oxygen tension than in the PBS treated gland. A hypoxic condition in the MECs at early lactation was demonstrated in our previous study (Shao *et al*., 2014). We suspect that the improved hypoxic condition by LPS treatment observed in this study at 12 and 24 h was probably due to 1) increased blood supply to the region with inflammation, and 2) reduced oxygen utilization in MECs resulted from a metabolic shift to anaerobic metabolism and reduced milk synthesis. LPS has been shown to increase the expression of vascular endothelial growth factor in MECs *in vitro* and *in vivo* (Minuti *et al*., 2015; Li *et al*., 2014; Devraj *et al*., 2017). However, mRNA expression of *Hif1α* and its target gene *Slc2a1* was not affected by LPS treatment. This was probably because the function of HIF-1α is mainly regulated at the protein level (Masoud *et al*., 2015; Devraj *et al*., 2017). There was a dramatic increase of GLUT1 expression at 24 h compared to 12 h in both treatment groups. This large increase over time may point to a potential effect of milk stasis as milk removal has not occurred in 24 h in these animals. This may result in cell differentiation towards autophagic behaviors, which promotes the expression of GLUT1 on the cell surface (Roy *et al*., 2017).

In this study, mRNA or protein expression of Csn2 and Lalba were significantly decreased at 12 and 24 h by LPS treatment. Although the mRNA expression of α-S1-caisin was elevated by LPS at 24 h, its protein expression was significantly inhibited by LPS at 24 h. These observations indicated that LPS inhibits milk protein synthesis in the mammary gland. The down-regulation of *Csn2* and *Lalba* transcription by LPS is

possibly mediated by LPS-induced NF-κβ and cytokine production. It has been shown that LPS treatment of bovine MECs decreases phosphorylation of mTORC1 and its targets like ribosomal S6 kinase 1 (Liu *et al*., 2015), which leads to the activation of NF-κβ (Zhang *et al*., 2016). NF-κβ activation has also been shown in previous mouse mastitis models (He *et al*., 2015; Jiang *et al*., 2018). NF- κβ activation has been shown to decrease prolactin receptor mediated STAT5a phosphorylation, and the *Csn2* gene promoter in rodents and cattle have a consensus NF- κβ binding sequence that overlaps the STAT5a binding site (Doppler *et al*., 2002; Geymayer *et al*., 2000; Malewski *et al*., 1995). The decrease in phosphorylation of mTORC1 and its targets like ribosomal S6 kinase 1 can also inhibit the overall gene translation. Moreover, reduced Lalba expression can result in milk yield decrease because of its role in regulating lactose synthesis which control milk osmolality (Habib *et al*., 2017).

In summary, LPS challenge greatly induced inflammation in the mammary gland. Expression of CC3 and Bax pointed to a caspase 3 activation and potential increase in cell apoptosis by LPS, but apoptotic signals were greatly reduced by 24 h. In addition, a oxidative stress was transiently increased by LPS infusion at 12 h, accompanied by enhanced expression of Nrf2 targeted antioxidative genes. Furthermore, LPS inhibited milk protein gene expression, but improved hypoxic condition in the mammary gland. These observations suggest that increased cell apoptosis due to oxidative stress, changed MEC metabolism, and reduced milk protein gene expression may all contribute to the decrease in milk production during mastitis.

Acknowledgement: We would like to acknowledge Dr. Ruth Blauwiekel and the animal care staff at the University of Vermont (UVM) Small Animal Facility for help in animal work, Dr. Alan Howard for statistical assistance, the members of the UVM Microscopy and Imaging Center for help in imaging analysis, and Dr. Scott Tighe and the members of the UVM Cancer Center for help in gene expression analysis. This project was supported by Agriculture and Food Research Initiative Competitive Grant no. 2016- 09363 from the USDA National Institute of Food and Agriculture and the Hatch/Multistate Project (#NE1748) of USDA.

2.6. References

1. Aerni-Flessner L, Abi-Jaoude M, Koenig A, Payne M, Hruz P. GLUT4, GLUT1, and GLUT8 are the dominant GLUT transcripts expressed in the murine left ventricle. Cardiovascular Diabetology (2012), 11; 63
2. Agarwal A, Hill-Kapturczak N, Nick HS. Heme Oxygenase-1, Inflammation and Atherosclerosis. Inflammatory and Infectious Basis of Atherosclerosis; 141-158
3. Aref NEM, Sayed AS, Zahran AM, Abdelaal GA, Nasser EA. Flow cytometric analysis of somatic cells and oxidant/antioxidant profile in dairy cows with sub-clinical mastitis. Bulgarian Journal of Veterinary Medicine (2018), 21(3); 347–357.
4. Aslantaş Ö, Demir C. Investigation of the antibiotic resistance and biofilm-forming ability of Staphylococcus aureus from subclinical bovine mastitis cases. Journal of Dairy Science (2016), 99(11), 8607-8613.
5. Atakisi O, Oral H, Atakisi E, Merhan O, Pancarci SM. Ozcan A, Marasli S, Polat B, Colak A, Kaya S. Subclinical mastitis causes alterations in nitric oxide, total oxidant and antioxidant capacity in cow milk. Research in Veterinary Science (2010), 89(1); 10-13
6. Baldwin VI R, Capuco A, Evock-Clover C, Grossi P, Choudhary R, Vanzant E, Elsasser T, Bertoni G, Trevisi E, Aiken GE, McLeod KR. Consumption of endophyte-infected fescue seed during the dry period does not decrease milk production in the following lactation. Journal of Dairy Science (2016), 99 (9), 7574-7589
7. Bhattarai D, Worku T, Dad R, Rehman ZU, Gong X, Zhang S. Mechanism of pattern recognition receptors (PRRs) and host pathogen interplay in bovine mastitis. Microbial Pathogenesis (2018), 120; Pages 64-70
8. Biswal S, Rizwan H, Pal S, Sabnam S, Parida P & Pal A. Oxidative stress, antioxidant capacity, biomolecule damage, and inflammation symptoms of sickle cell disease in children, Hematology (2019), 24(1); 1-9
9. Boscos C, Stefanakis A, Alexopoulos C, Samartzi F. Prevalence of subclinical mastitis and influence of breed, parity, stage of lactation and mammary bacteriological status on Coulter Counter Counts and California Mastitis Test in the milk of Saanen and autochthonous Greek goats. Small Ruminant Research (1996), 21(2); 139-147
10. Breyne K, De Vliegher S, De Visscher A, Piepers S, Meyer E. Technical note: A pilot study using a mouse mastitis model to study differences between bovine associated coagulase-negative staphylococci. Journal of Dairy Science (2015), 98(2); 1090-1100
11. Carter ME, Brunet A. FOXO transcription factors. Current Biology (2008), 17(4).
12. Chandler RL. Experimental bacterial mastitis in the mouse. Journal of Medical Microbiology (1970); 3; 273–282

13. Chen CM, Wu CT, Chiang CK, Liao BW, Liu SH. C/EBP homologous protein (CHOP) deficiency aggravates hippocampal cell apoptosis and impairs memory performance. PLoS One (2012), 7(7); e40801

14. Ci X, Zhou J, Lv H, Yu Q, Peng L, and Hua S. Betulin exhibits anti-inflammatory activity in LPS-stimulated macrophages and endotoxin-shocked mice through an AMPK/AKT/Nrf2-dependent mechanism., Cell Death and Disease (2017), 8, e2798
15. Devraj G, Beerlage C, Volkhard BB, Kempf AJ. Hypoxia and HIF-1 activation in bacterial infections. Microbes and Infections (2017) 19; 144-156
16. Doppler W, Geymayer S, Weirich HG. Synergistic and Antagonistic Interactions of Transcription Factors in the Regulation of Milk Protein Gene Expression. In: Mol J.A., Clegg R.A. (eds) Biology of the Mammary Gland. Advances in Experimental Medicine and Biology (2002), 480. Springer, Boston, MA
17. Dürr JW, Cue RI, Monardes HG, Moro-Méndez J, Wade KM. Milk losses associated with somatic cell counts per breed, parity and stage of lactation in Canadian dairy cattle. Livestock Science (2008), 117 (2–3); 225-232

18. Elazar S, Gonen E, Livneh-Kol A, Rosenshine I, Shpigel NY. Essential role of neutrophils but not mammary alveolar macrophages in a murine model of acute Escherichia coli mastitis. *Veterinary Res*earch (2010) 41(4); 53

19. Endo M, Mori M, Akira S, Gotoh T. C/EBP Homologous Protein (CHOP) Is Crucial for the Induction of Caspase-11 and the Pathogenesis of Lipopolysaccharide-Induced Inflammation. Journal of Immunology (2006), 176; 6245-6253
20. Fan WJ, Li HP, Zhu HZ, Sui SP, Chen PG, Deng Y, Sui TM, Wang YY. NF-κB is involved in the LPS-mediated proliferation and apoptosis of MAC-T epithelial cells as part of the subacute ruminal acidosis response in cows. Biotechnology Letters (2016), 38 (11); 1839–1849
21. Fitzpatrick SF, Gojkovic M, Macias D, Tegnebratt T, Li Lu L, Samén E, Rundqvist H, Johnson RS. Glycolytic Response to Inflammation Over Time: Role of Myeloid HIF-1 alpha. Frontiers in Physiology (2018), 9(1624); 1-7.
22. Gallo-Oller G, Ordoñez R, Dotor J. A new background subtraction method for Western blot densitometry band quantification through image analysis software. Journal of Immunological Methods (2018) 457; 1-5
23. Gao X, Zhang Z, Li Y, Hu X, Shen P, Fu YH, Cao Y, Zhang N. Selenium Deficiency Deteriorate the Inflammation of S. aureus Infection via Regulating NF-κB and PPAR-γ in Mammary Gland of Mice. Biological Trace Element Research (2016), 172(1); 140–147
24. Ge X, Wang L, Li M, Xu N, Yu F, Yang F, Li R, Zhang F, Zhao B, and Du J. Vitamin D/VDR signaling inhibits LPS-induced IFNγ and IL-1β in Oral epithelia by regulating hypoxia-inducible factor-1α signaling pathway. Cell Communication and Signaling (2019) 17:18

25. Geymayer S, Doppler W. Activation of NF-κB p50/p65 is regulated in the developing mammary gland and inhibits STAT5-mediated β-casein gene expression. The FASEB Journal 2000 14:9, 1159-1170
26. Gogoi-Tiwari J, Williams V, Waryah CB, Costantino P, Al-Salami H, Mathavan S. Mammary Gland Pathology Subsequent to Acute Infection with Strong versus Weak Biofilm Forming Staphylococcus aureus Bovine Mastitis Isolates: A Pilot Study Using Non-Invasive Mouse Mastitis Model. PLoS ONE (2017), 12(1): e0170668.
27. Gogoi-Tiwari J, Williams V, Waryah CB, Eto KY, Tau M, Costantino P, Tiwari HK, Mukkur T. Comparative studies of the immunogenicity and protective potential of biofilm vs planktonic Staphylococcus aureus vaccine against bovine mastitis using non-invasive mouse mastitis as a model system. Biofouling (2015), 31(7); 543-554
28. Greer SN, Metcalf JL, Wang Y, Ohh M. The updated biology of hypoxia-inducible factor. The EMBO Journal (2012): 31(11).
29. Guimarães JLB, Brito MAVP, Lange CC2, Silva MR, Ribeiro JB, Mendonça LC, Mendonça JFM, Souza GN. Estimate of the economic impact of mastitis: A case study in a Holstein dairy herd under tropical conditions. Preventative Veterinary Medicine (2017), 142; 46-50.
30. Günther J, Czabanska A, Bauer I, Leigh JA, Holst O, Seyfert HM. Streptococcus uberis strains isolated from the bovine mammary gland evade immune recognition by mammary epithelial cells, but not of macrophages. Veterinary Research (2016), 47(13)
31. Habib M, Hazari YM, Iqbal ST, Ganai TAS, Sheikh FD, Andrabi KI, Fazili KM. Novel variations in 5′ flanking region of the α-Lactalbumin gene in exotic and indigenous cattle. Meta Gene (2017), 13; 149-153
32. Hagiwara S, Mori K, Nagahata H. Predictors of fatal outcomes resulting from acute Escherichia coli mastitis in dairy cows, Journal of Veterinary Medical Science (2016), 78(5); 905-908
33. Han LQ, Zhou Z, Ma Y, Batistel F, Osorio JS, Loor JJ. Phosphorylation of nuclear factor erythroid 2-like 2 (NFE2L2) in mammary tissue of Holstein cows during the periparturient period is associated with mRNA abundance of antioxidant gene networks. Journal of Dairy Science (2018) 101(7); 6511-6522
34. He X, Wei Z, Zhou E, Chen L, Kou J, Wang J, Yang Z. Baicalein attenuates inflammatory responses by suppressing TLR4 mediated NF-κB and MAPK signaling pathways in LPS-induced mastitis in mice. International Immunopharmacology (2015), 28(1); 470-476
35. Hinthong W, Pumipuntu N, Santajit S, Kulpeanprasit S, Buranasinsup S, Sookrung N, Chaicumpa W, Aiumurai P, Indrawattana N. Detection and drug resistance profile of Escherichia coli from subclinical mastitis cows and water supply in dairy farms in Saraburi Province, Thailand. PeerJ (2017), 5; e3431

36. Hoeben D, Burvenich C, Trevisi E, Bertoni G. Role of endotoxin and TNF-α in the pathogenesis of experimentally induced coliform mastitis in periparturient cows. Journal of Dairy Research (2000), 67(4); 503-514

37. Hosoya KI, Tomi M, Ohtsuki S, Takanaga H, Saeki S, Kanai Y, Endou H, Naito M, Tsuruo T, Teraski T. Enhancement of l-Cystine Transport Activity and Its Relation to xCT Gene Induction at the Blood-Brain Barrier by Diethyl Maleate Treatment. Journal of Pharmacology and Experimental Therapeutics (2002), 302 (1); 225-231
38. Hwan S, Bao Y, Vasilis L, Androutsopoulos P, Tsukamotoc C, Shin TS, Tsatsakis AM, Chunga G. Short Communication: Anti-inflammatory effects of soyasapogenol I-αa via downregulation of the MAPK signaling pathway in LPS-induced RAW 264.7 macrophages. Food and Chemical Toxicology (2018), 113; 211-217
39. Janzen JJ. Economic Losses Resulting from Mastitis. A Review. Journal of Dairy Science (1970), 53(9); 1151–1160
40. Jia R, Du J, Cao L, Li Y, Johnson O, Gu Z, Jeney G, Xu P, Yin G. Antioxidative, inflammatory and immune responses in hydrogen peroxide-induced liver injury of tilapia (GIFT, Oreochromis niloticus). Fish and Shellfish Immunology (2019), 84; 894-905
41. Jiang K, Ma X, Guo S, Zhang T, Zhao G, Wu H, Wang X, Deng G. Anti-inflammatory Effects of Rosmarinic Acid in Lipopolysaccharide-Induced Mastitis in Mice. Inflammation (2018) 41(2); 437-448
42. Jiang Q, Chen S, Ren W, Liu G, Yao K, Wu G, Yin Y. Escherichia coli aggravates endoplasmic reticulum stress and triggers CHOP-dependent apoptosis in weaned pigs. Amino Acids (2017), 49(12); 2073–2082

43. Jin X, Wang K, Liu L, Liu H, Zhao FQ, Liu JX. Nuclear factor-like factor 2-antioxidant response element signaling activation by tert-butylhydroquinone attenuates acute heat stress in bovine mammary epithelial cells Journal of Dairy Science (2016), 99(11); 9094-9103

44. Klotz LO, Steinbrenner H. Cellular adaptation to xenobiotics: Interplay between xenosensors, reactive oxygen species and FOXO transcription factors. Redox Biology (2017), 13; 646-654
45. Kobayashi, K., Oyama, S., Uejyo, T., Kuki, C., Rahman, M. M., & Kumura, H. Underlying mechanisms involved in the decrease of milk secretion during Escherichia coli endotoxin induced mastitis in lactating mice. Veterinary research (2013), 44(1); 119.
46. Laukka T, Mariani CJ, Ihantola T, Cao JZ, Hokkanen J, Kaelin Jr WG, Godley LA, and Koivunen P. Fumarate and Succinate Regulate Expression of Hypoxia-inducible Genes via TET Enzymes. Journal of Biological Chemistry (2016), 291, 4256-4265.

47. Lee B, Kwon E, Kim Y, Hee J, Wook KS, Jin S., Lee K, Won D, won Sohn KJ, Kima TH, Jid JD. 1α,25-Dihydroxyvitamin D3 upregulates HIF-1 and TREM-1 via mTOR signaling. Immunology Letters (2015), 163(1); 14-21
48. Leitner G, Chaffer M, Shamay A, Shapiro F, Merin U, Ezra E, Saran A, Silanikove N. Changes in Milk Composition as Affected by Subclinical Mastitis in Sheep. Journal of Dairy Science (2004), 87(1);46-52.
49. Lewerenz J, Maher P, Methner A. Regulation of xCT expression and system x−c function in neuronal cells. Amino Acids (2012), 42(1); 171-179
50. Li C, Yang F, Liu F, Li D, Yang T. NRF2/HO-1 activation via ERK pathway involved in the anti-neuroinflammatory effect of Astragaloside IV in LPS induced microglial cells. Neuroscience Letters (2018), 666 (14), 104-11
51. Li F, Wang W, Cao Y, Liang D, Zhang W, Zhang Z, Jiang H, Guo M, Zhang N. Inhibitory effects of astragalin on lipopolysaccharide-induced inflammatory response in mouse mammary epithelial cells. Journal of Surgical Research (2014), 192; 573-581
52. Malewski T, Zwierzchowski L. Computer-aided analysis of potential transcription-factor binding sites in the rabbit P-casein gene promoter. BioSystems (1995), 36; 109-l19.
53. Magro G, Brevini TAL, Maglie MD, Minozzi G, Scanziani E, Piccinin R. An explant of heifer mammary gland to study the immune response of the organ. Research in Veterinary Science (2017), 44-50
54. Mandal, PK, Seiler A, Perisic T, Kölle P, Canak AB, Förster H, Conrad M. (2010). System x(c)- and thioredoxin reductase 1 cooperatively rescue glutathione deficiency. The Journal of biological chemistry, 285(29); 22244–22253
55. Masoud GN, Li W. HIF-1α pathway: role, regulation and intervention for cancer therapy. Acta Pharmaceutica Sinica B (2015), 5(5) 378-389
56. Matsunaga K, Tsugami Y, Kumai A, Suzuki T, Nishimura T, Kobayashi K. IL-1β directly inhibits milk lipid production in lactating mammary epithelial cells concurrently with enlargement of cytoplasmic lipid droplets. Experimental Cell Research (2018), 370 (2); 365-372
57. McFadden T, Akers R, Capuco A. Relationship of Milk Proteins in Blood with Somatic Cell Count in Milk in Dairy Cows. Journal of Dairy Science (1988), 71; 826-834
58. Memon MA, Wang Y, Xu T, Ma N, Zhang H, Roy AC, Aabdin ZU. Lipopolysaccharide induces oxidative stress by triggering MAPK and Nrf2 signaling pathways in mammary glands of dairy cows fed a high-concentrate diet. Microbial Pathogenesis (2019), 128; 268-275
59. Minuti A, Zhou Z, Graugnard DE, Rodriguez-Zas SL, Palladino AR, Cardoso FC, Trevisi E, Loor JJ. Acute mammary and liver transcriptome responses after an intramammary Escherichia coli lipopolysaccharide challenge in postpartal dairy cows. Physiological Reports (2015), 3(4); e12388.

60. Mir BA, Razak R, Ali A, Muzamil S, Mir MR, Khaliq T, Baba OK, Rehman MU, Bilal S, Hussain I, Rashid IM. Assessment of antioxidant profile in subclinical and clinical mastitis in dairy cattle. Journal of Entomology and Zoology Studies (2017), 5(6); 1022-1025
61. Neville M, Monks J. The Cell Biology of the Lactating Epithelium. Reference Module in Biomedical Research: Encyclopedia of Reproduction Second Edition (2018), 2; 779-785
62. Nguyen T, Sherratt PJ, and Pickett CB. Regulatory Mechanisms Controlling Gene Expression Mediated by the Antioxidant Response Element. Annual Review of Pharmacology and Toxicology (2003), 43(1); 233-260
63. Nishitoh H. CHOP is a multifunctional transcription factor in the ER stress response. The Journal of Biochemistry (2012), 151(3); 217–219
64. Notebaert S, Meyer E. Mouse models to study the pathogenesis and control of bovine mastitis. A review, Veterinary Quarterly (2006), 28(1); 2-13
65. Oyadomari S, Mori M. Roles of CHOP/GADD153 in endoplasmic reticulum stress. Cell Death and Differentiation (2004), 11; 381–389
66. Påhlman S, Lund LR, Jögi A (2015) Differential HIF-1α and HIF-2α Expression in Mammary Epithelial Cells during Fat Pad Invasion, Lactation, and Involution. PLoS ONE 10(5): e0125771.
67. Rabaneda-Lombarte N, Xicoy-Espaulella E, Serratosa J, Saura J, Solà C, Parkinsonian Neurotoxins Impair the Pro-inflammatory Response of Glial Cells. Frontiers in molecular neuroscience (2019), 11, 479. doi:10.3389/fnmol.2018.00479
68. Ross D, Siegel D. Functions of NQO1 in Cellular Protection and CoQ10 Metabolism and its Potential Role as a Redox Sensitive Molecular Switch. Frontiers in physiology (2017), 8; 595.
69. Roy S, Leidal AM, Ye J, Ronen SM, Debnath J. Autophagy-Dependent Shuttling of TBC1D5 Controls Plasma Membrane Translocation of GLUT1 and Glucose Uptake. Molecular Cell (2017), 67(1); 84-95.e5
70. Ryter SW, Choi AMK. Targeting heme oxygenase-1 and carbon monoxide for therapeutic modulation of inflammation. Translational Research (2016), 167(1); 7-34
71. Santos G, Bottino MP, Santos APC, Simões LMS, Souza JC, Ferreira MBD, da Silveira JC, Ávila ACFCM, Bride A, Sales JNS. Subclinical mastitis interferes with ovulation, oocyte and granulosa cell quality in dairy cows. Theriogenology (2018), 119; 214-219
72. Schweikl H, Gallorini ML, Pöschl G, Vera, Urmanna V, Petzel C, Bolay C, Hiller KA, Cataldi A, Buchall W. Functions of transcription factors NF-κB and Nrf2 in the inhibition of LPS-stimulated cytokine release by the resin monomer HEMA. Dental Materials (2018), 34(11); 1661-1678

73. Seegers H, Fourichon C, Beaudeau F. Production effects related to mastitis andmastitis economics in dairy cattle herds. Veterinary Research, BioMed Central (2003), 34(5): 475-91.
74. Shao Y, Zhao FQ. Emerging evidence of the physiological role of hypoxia in mammary development and lactation. Journal of Animal Science and Biotechnology (2014), (5):9.
75. Shao Y, Wellman TL, Lounsbury KM, and Zhao FQ. Differential regulation of GLUT1 and GLUT8 expression by hypoxia in mammary epithelial cells. American Journal of Physiology Regulation and Integrated Comparative Physiology (2014), 307; R237–R247
76. Shi HY, Yan SM, Guo YM, Zhang BQ, Guo XY, Shi BL. Vitamin A pretreatment protects NO-induced bovine mammary epithelial cells from oxidative stress by modulating Nrf2 and NF-κB signaling pathways, Journal of Animal Science (2018), 96(4); 1305–1316
77. Silanikove N, Rauch-Cohen A, Shapiro F, Blum S, Arieli A, Leitner G. Lipopolysaccharide challenge of the mammary gland in bovine induced a transient glandular shift to anaerobic metabolism. Journal of Dairy Science (2011), 94(9); 4468-4475
78. Skelly DT, Hennessy E, Dansereau M-A, Cunningham C. A Systematic Analysis of the Peripheral and CNS Effects of Systemic LPS, IL-1B, TNF-α and IL-6 Challenges in C57BL/6 Mice. PLoS ONE (2013), 8(7); e69123.
79. Talwar H, Bauerfeld C, Bouhamdan M, Farshi P, Liu Y, Samavati L. MKP-1 negatively regulates LPS-mediated IL-1β production through p38 activation and HIF-1α expression. Cellular Signaling (2017), 34; 1-10
80. Talwar H, Bouhamdan M, Bauerfeld C, Talreja J, Aoidi R, Houde N, Charron J, Samavati L. MEK2 Negatively Regulates Lipopolysaccharide-Mediated IL-1b Production through HIF-1a Expression. The Journal of Immunology (2019), 202(6); 1815-1825
81. Tanaka KAK, Kurihara S, Shibakusa T, Chiba Y, Mikami T. Cystine improves survival rates in an LPS-induced sepsis mouse model. Clinical Nutrition (2015), 34(6); 1159-1165
82. Tanaka A, To J, O'Brien B, Donnely S, Lund M. Selection of reliable reference genes for the normalisation of gene expression levels following time course LPS stimulation of murine bone marrow derived macrophages. Immunology (2017), 18(43).
83. Vanderhaeghen W, Cerpentier T, Adriaensen C,Vicca J, Hermans K, Butaye P; Methicillin-resistant Staphylococcus aureus (MRSA) ST398 associated with clinical and subclinical mastitis in Belgian cows. Veterinary Microbiology (2010), 144; 166–171
84. Védrine M, Berthault C, Leroux C, Répérant-Ferter M, Gitton C, Barbey S. Sensing of Escherichia coli and LPS by mammary epithelial cells is modulated by O-antigen chain and CD14. PLoS ONE (2018), 13(8); e0202664

85. Wang JJ, Wei ZK, Zhang X, Wang YN, Fu YH, Yang ZT. Butyrate protects against disruption of the blood-milk barrier and moderates inflammatory responses in a model of mastitis induced by lipopolysaccharide. British Journal of Pharmacology (2017), 174(21); 3811–3822
86. Wible RS, Tran QT, Fathima S, Sutter CH, Kensler TW, Sutter TR. Pharmacogenomics of Chemically Distinct Classes of Keap1-Nrf2 Activators Identify Common and Unique Gene, Protein, and Pathway Responses In Vivo. Molecular Pharmacology (2018), 93(4); 297-308
87. Wilson DJ, Stewart KN, Sears PM. Effects of stage of lactation, production, parity and season on somatic cell counts in infected and uninfected dairy goats. Small Ruminant Research Volume (1995), 16(2); 165-169
88. Xiao HB, Wang CR, Liu ZK, Wang JY. LPS induces pro-inflammatory response in mastitis mice and mammary epithelial cells: Possible involvement of NF-κB signaling and OPN. Pathologie Biologie (2015), 63(1); 11-16
89. Yang W, Li H, Cong X, Wang X, Jiang Z, Zhang Q, Qi X, Gao S, Cao R, Tian W. Baicalin attenuates lipopolysaccharide induced inflammation and apoptosis of cow mammary epithelial cells by regulating NF-κB and HSP72. International Immunopharmacology (2016), 40; 139-145
90. Yeligar SM, Machida K, Kalra VK. Ethanol-induced HO-1 and NQO1 Are Differentially Regulated by HIF-1and Nrf2 to Attenuate Inflammatory Cytokine Expression. The Journal of Biological Chemistry (2010), 285(46); 35359–35373
91. Zheng J, Watson AD, Kerr DE. Genome-Wide Expression Analysis of Lipopolysaccharide-Induced Mastitis in a Mouse Model. Infection and Immunity (2006), 74(3); 1907-1915

Table 1: Primers used in reverse transcription-PCR

Gene Symbol	GenBank Accession Number	Product Length (bp)	Primer Sequence (5'→3')*	Reference
Csn1s1	NM_007784.3	193	F: CCTTTCCCCTTTGGGCTTAC R: TGAGGTGGATGGAGAATGGA	Kobayashi *et al*., 2013
Csn2	NM_001286022.1	330	F: CTTCAGAAGGTGAATCTCATGGG R: CAGATTAGCAAGACTGGCAAGG	
Gapdh	NM_001289726.1	134	F: GAGCGAGACCCCACTAACATC R: GCGGAGATGATGACCCTTTT	
Lalba	NM_010679.1	106	F: ACCAGTGGCTACGACACAC R: CGGGGAACTCACTACTTTTACAC	
Slc2a1	NM_011400.3	361	F: CTTCGCCCTGGCCCTGCAGGAGC R: GGCACCCCCCTGCCGGAAGCCGGA	Rabaneda-Lombarte *et al*., 2018
Actb1	NM_007393.5	334	F: TGGAATCCTGTGGCATCCA R: TAACAGTCCGCCTAGAAGCA	
Hprt	NM_013556.2	76	F: CCCCAAAATGGTTAAGGTTGC R: AACAAAGTCTGGCCTGTATCC	Aerni-Flessner *et al*., 2012
IL-1β	NM_008361.4	69	F: GCACACCCACCCTGCA R: ACCGCTTTTCCATCTTCTTCTT	Skelly *et al*., 2013
IL-6	NM_001314054.1	73	F: TCCAGAAACCGCTATGAAGTTC R: CACCAGCATCAGTCCCAAGA	
TNFA	NM_001278601.1	149	F: CTCCAGGCGGTGCCTATG R: GGGCCATAGAACTGATGAGAGG	
Bcl-2	NM_009741.5	205	F: GTGGTGGAGGAACTCTTCAG R: GTTCCACAAAGGCATCCCAG	Xue *et al*., 2016
Cycs	NM_007808.5	133	F: GAGGCAAGCATAAGACTGGA R: TACTCCATCAGGGTATCCTC	
Hif1a	NM_001313920.1	135	F: GCTTACACACAGAAATGGCC R: AGCACCTTCCACGTTGCTGA	

Casp3	NM_009810.3	226	F: CCTCAGAGAGACATTCATGG R: GCAGTAGTCGCCTCTGAAGA	
Casp9	NM_001277932.1	152	F: AGTTCCCGGGTGCTGTCTAT R: GCCATGGTCTTTCTGCTCAC	
Bax	NM_007527.3	156	F: AGTGATGGACGGGTCCGGGG R: GGCGGCTGCTCCAAGGTCAG	
Bid	NM_007544.4	96	F: TCTGAGGTCAGCAACGGTTC R: CTCTTGGCGAGTACAGCCAG	Jia *et al*., 2016
Chop	NM_134248.2	260	F: ATGCCCATCTTCTGCTTGTCA R: CCTTGTAGTTGTGGGTCTTGT	Chen *et al*., 2012
Nrf2	NM_001011167 8.2	94	F: GCAGAGACATTCCCGTTTGT R: CCTGAGGAGGAGCAGTGAAG	Shi *et al*., 2018
Nqo1	NM_008706.5	424	F: TCACAGGGGAGCCGAAGGACT R: GGGGTGTGGCCAATGCTGTA	Wang *et al*., 2017
Hox1	NM_022994.3	270	F: GCTCTATCGTGCTCGCATGA R: AATTCCCACTGCCACGGTC	
xCt	NM_011990.2	182	F: CCTGGCATTTGGACGCTACAT R: TCAGAATTGCTGTGAGCTTGCA	Hosoya *et al*., 2002
hnRNPAB	NM_001048061.1	204	F: TTTGGCGAGTTTGGGGAGATT R:GCCATACTGCTGCTGCTGATAGAC	Tanaka *et al*., 2017
Stx5a	NM_001167799.1	205	F: CGGGATCGGACCCAGGAGTTC R: CAAAGAGGGACTTGCGCTTTG	

* F = Forward primer, R = reverse primer.

Table 2: Statistical analysis of the mRNA expression and other variables.

mRNA expression or other variables	Treatment	Time	Time x Treatment	12 h Treatment	24 h Treatment
Bcl2	0.363	0.551	0.530	0.823	0.311
Bid	0.293	0.090	0.881	0.410	0.499
Csn1s1	0.058	0.013	0.061	0.982	0.013
Csn2	0.001	0.164	0.607	0.022	0.004
CytS	0.539	0.334	0.270	0.246	0.704
Gadd153	0.040	0.054	0.012	0.559	0.006
Hif1a	0.315	0.211	0.165	0.138	0.650
Il1B	<0.001	0.324	0.688	0.004	0.002
Il6	<0.001	0.314	0.412	<0.001	0.008
Lalba	0.017	0.233	0.440	0.016	0.013
Nqo1	0.571	0.711	0.035	0.05	0.240
Nrf2	0.834	0.616	0.075	0.244	0.153
Slc2a1	0.269	<0.001	0.607	0.674	0.238
Tnfa	0.003	0.506	0.598	0.046	0.011
xCt	0.026	0.001	0.855	0.117	0.076
CC3[1] protein	0.031	0.075	0.074	0.021	0.698
Csn2 protein	<0.001	0.088	0.561	0.0001	0.006
Csn1s1 protein	0.007	0.003	0.007	0.992	<0.001
Hypoxyprobe staining density	<0.001	<0.001	<0.001	<0.001	<0.001
TAC[2]	0.011	0.411	0.858	0.067	0.061
H_2O_2[3]	0.935	0.049	<0.001	<0.001	<0.001

[1] CC3 = cleaved caspase 3, [2] TAC = total antioxidative capacity, [3] Hydrogen peroxide level,

Table 3: Statistical analysis of the expression of markers that presented side difference.

mRNA expression or other variable	Treatment	Side	Time	Time* Treatment	12 h Treatment	24 h Treatment
Bax	0.004	0.003	0.507	0.026	<0.001	0.467
Casp3	0.521	0.006	0.083	0.123	0.127	0.495
Casp9	0.592	0.005	0.080	0.405	0.835	0.319
Hox1	0.488	0.002	0.267	0.035	0.239	0.056
Lalba Protein	0.005	0.009	0.820	0.518	0.001	0.003

Figure Legend

Figure 1: Liposaccharide (LPS) Challenge Incurs Mammary Gland Inflammation. The left and right sides of fourth mammary gland are alternatively injected with LPS or PBS through teats at day 3 of lactation. The injected glands are individually collected after 12 or 24 h of injection (n=8). A-D: Hematoxylin and Eosin staining of formalin-fixed, paraffin embedded mammary tissue sections of 12 h (A: PBS; B: LPS) and 24 h (C: PBS; D: LPS). E-H: mRNA expression of cytokines Interleukin-1β (E: *Il1B*), Interleukin-6 (F: *Il6*), and Tumor Necrosis Factor-α (G: *Tnfa*) by real-time quantitative PCR. Relative gene expression of cytokines is calculated by 2^^(-ddCt) method and normalized by the mRNA levels of the internal control housekeeping genes *ActinB*, *GAPDH*, *HRPT*, *Stx5a* and *hnRNPAB.* Error bars represent standard error, and different letters above each bar indicate significant differences. Significance is declared when ($P < 0.05$).

Figure 2. Liposaccharide (LPS) Treatment Induced Apoptosis in the Mammary Gland. Mammary tissues treated with PBS or LPS for either 12 or 24 h (n=8) are collected and analyzed by real-time PCR for mRNA expression (A, D-I) or western blot for protein levels (B: representative blots with animal numbers, C: quantitative representation). Caspase 3 (A: *Casp3*), cleaved caspase 3 (B: CC3), Caspase 9 (D: *Casp9*), Bcl-2-associated protein X (E: *Bax*), endoplasmic reticulum stress marker C/EBP Homologous Protein (F: CHOP; *GADD153*), (G) B-Cell Lymphoma 2 (G: *Bcl2),* (H) Cytochrome-C (I: *CytS*) and BH3-interacting domain (I: *Bid*) Relative mRNA expression is calculated by 2^^(-ddCt) method and normalized by expression levels of housekeeping genes *ActinB*, *GAPDH*, *HRPT*, *Stx5a* and *hnRNPAB.* Protein expression of CC3 was

normalized by GAPDH. Error bars represent standard error, and different letters above each bar indicate significant differences. Significance is declared when ($P < 0.05$).

Figure 3. Liposaccharide (LPS) Increases Oxidative Stress and Activates the Antioxidative Response in the Mammary Gland. Mammary tissues treated with PBS or LPS for either 12 or 24 h (n=8) are collected and analyzed for the concentration of hydrogen peroxide (A), total antioxidant capacity relative to antioxidant standard Trolox (B) and mRNA expression of Nuclear erythroid-related factor 2 (C: *Nrf2*), NAD(P)H quinone oxidase 1 (D: *Nqo1*), cysteine transporter *xCT*, (E), and Heme oxygenase 1 (F: *Hox1*). Gene expression is calculated by 2^^(-ddCt) method and normalized by the levels of housekeeping genes *ActinB*, *GAPDH*, *HRPT*, *Stx5a* and *hnRNPAB.* Error bars represent standard error, and different letters above each bar indicate significant differences. Significance is declared when ($P < 0.05$).

Figure 4. Effect on Liposaccharide (LPS) Treatment on Hypoxic Condition in the Mammary Gland. Mice are injected intraperitoneally with pimonidazole HCl, and mammary tissues are treated with PBS or LPS for 12 or 24 h (n=8). A-C: Representative immunostaining of hyproxyprobe in A: the LPS-treated gland and B: PBS-treated gland at 12 h. C: Negative control staining is performed by the omission of the first antibody against hypoxyprobe. D: Quantitative representation of relative hypoxic condition in LPS- and PBS-infused glands. E & F: mRNA expression of hypoxia-inducible factor 1 (*Hif1a*, E) and glucose transporter 1 (*Slc2a1*, F) by real-time PCR. Relative gene expression is calculated by 2^^(-ddCt) method and normalized by the expression levels of housekeeping genes *ActinB*, *GAPDH*, *HRPT*, *Stx5a* and *hnRNPAB*. Error bars represent

standard error, and different letters above each bar indicate significant differences. Significance is declared when ($P < 0.05$).

Figure 5. Liposaccharide (LPS) Challenge Reduces Milk Protein Expression in the mammary gland. Mammary tissues treated with PBS or LPS for 12 or 24 h (n=8) are collected and analyzed by real-time PCR for mRNA expression (A-C) and western blot for protein levels (D-F: quantitative presentation, G: representative images with animal numbers) of β-casein (A&D: *Csn2*), α-lactalbumin (B&E: *Lalba*), and α-S1-casein (C&F: *Csn1s1*). Relative mRNA expression is calculated by 2^^(-ddCt) method and normalized by the expression of housekeeping genes *ActinB, GAPDH, HRPT, Stx5a* and *hnRNPAB.* In D-F, relative protein levels of milk proteins are normalized by the levels of GAPDH (D). Error bars represent standard error, and different letters above each bar indicate significant differences. Significance is declared when ($P < 0.05$).

Figure 1

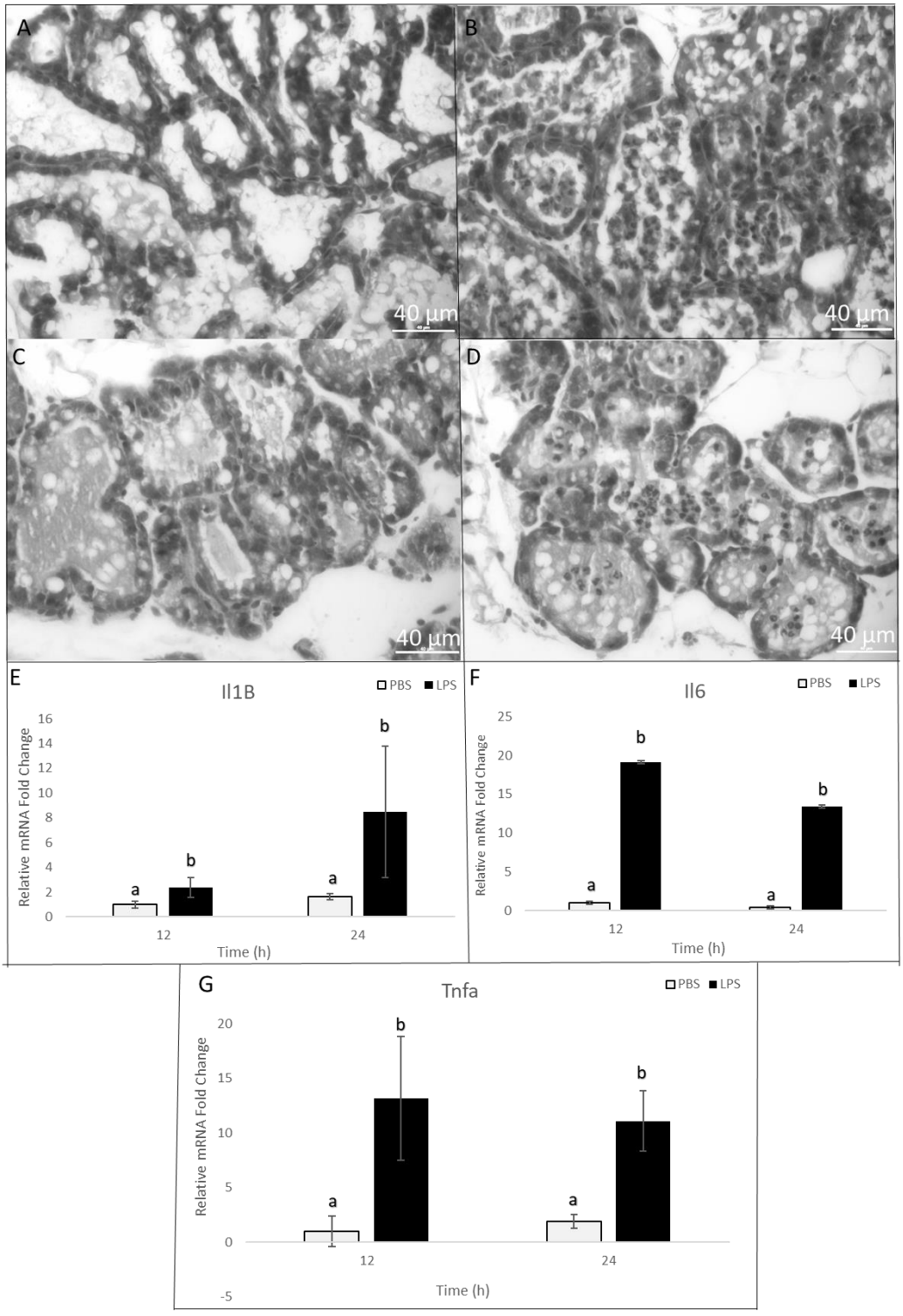

A
40 µm
B
40 µm
C
40 µm
D
40 µm
E
Il1B
PBS
LPS
Relative mRNA Fold Change
16
14
12
10
8
6
4
2
0
a
b
a
b
12
24
Time (h)
F
Il6
PBS
LPS
Relative mRNA Fold Change
25
20
15
10
5
0
a
b
a
b
12
24
Time (h)
G
Tnfa
PBS
LPS
Relative mRNA Fold Change
20
15
10
5
0
-5
a
b
a
b
12
24
Time (h)

Figure 2

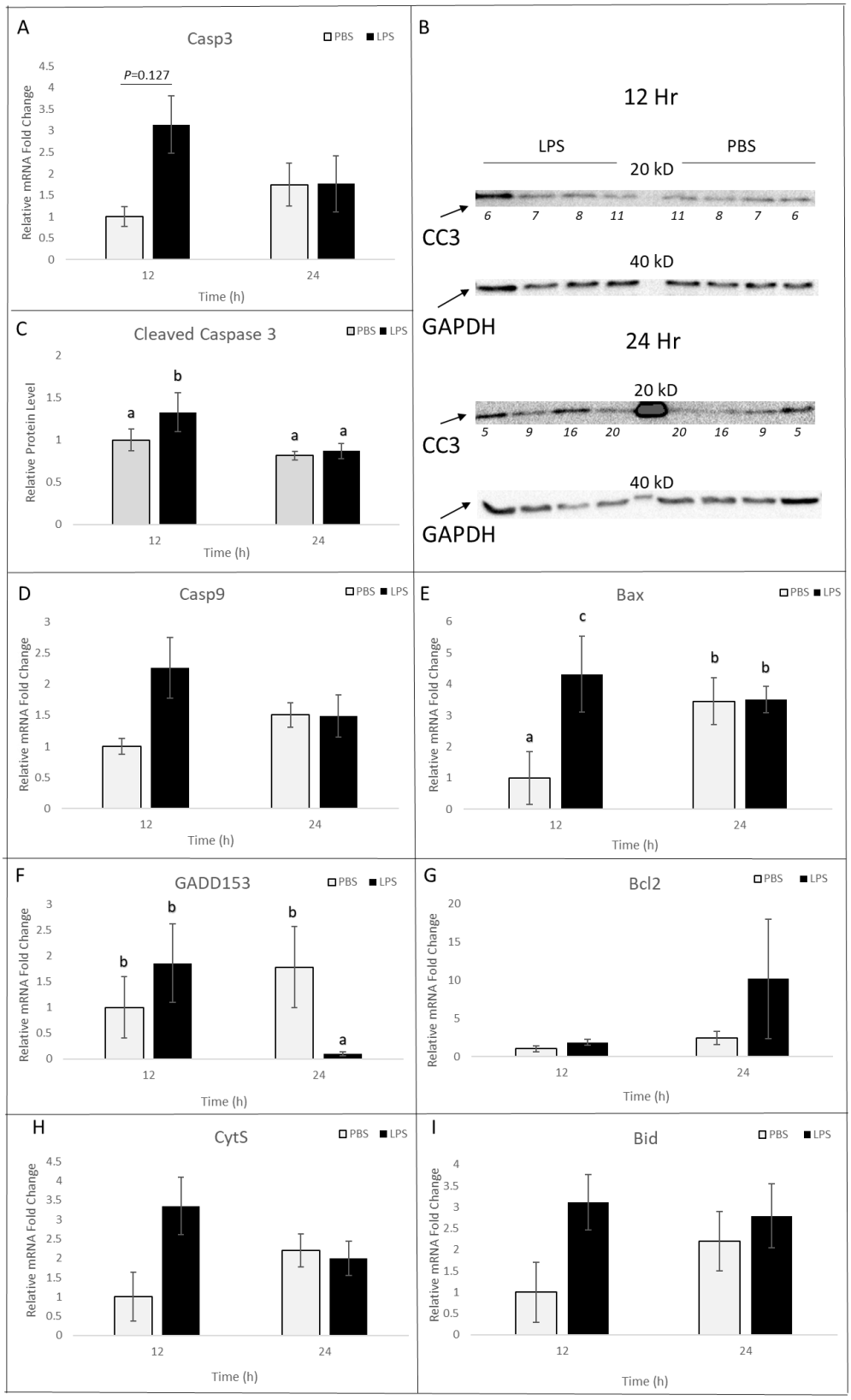

A
Casp3
PBS LPS
P=0.127
Relative mRNA Fold Change
Time (h)
B
12 Hr
LPS
PBS
20 kD
6 7 8 11 11 8 7 6
CC3
40 kD
GAPDH
24 Hr
20 kD
5 9 16 20 20 16 9 5
CC3
40 kD
GAPDH
C
Cleaved Caspase 3
Relative Protein Level
D
Casp9
E
Bax
F
GADD153
G
Bcl2
H
CytS
I
Bid

Figure 3

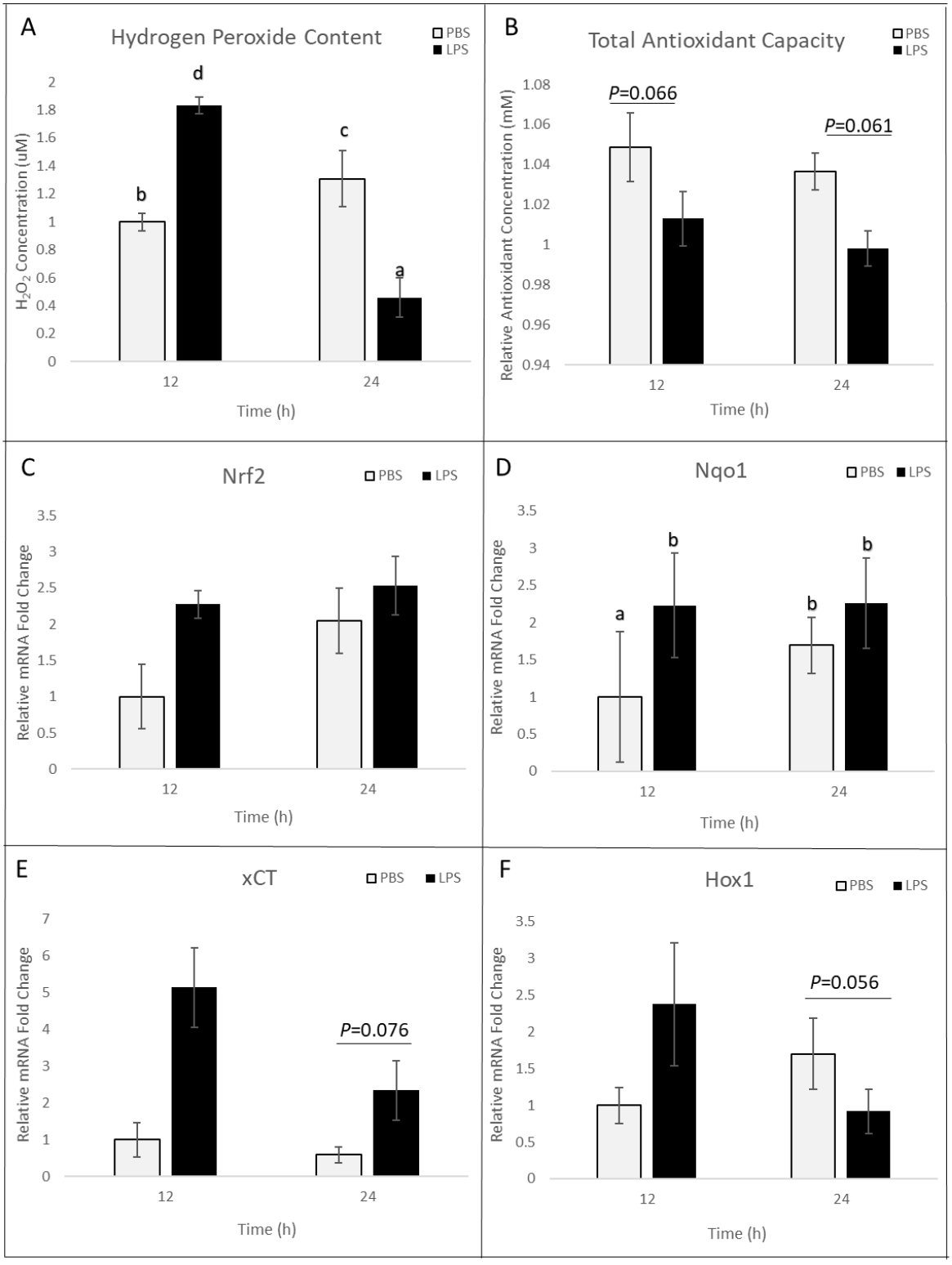

A
Hydrogen Peroxide Content
PBS
LPS
H_2O_2 Concentration (uM)
b
d
c
a
12
24
Time (h)
B
Total Antioxidant Capacity
PBS
LPS
Relative Antioxidant Concentration (mM)
P=0.066
P=0.061
12
24
Time (h)
C
Nrf2
PBS
LPS
Relative mRNA Fold Change
12
24
Time (h)
D
Nqo1
PBS
LPS
Relative mRNA Fold Change
a
b
b
b
12
24
Time (h)
E
xCT
PBS
LPS
Relative mRNA Fold Change
P=0.076
12
24
Time (h)
F
Hox1
PBS
LPS
Relative mRNA Fold Change
P=0.056
12
24
Time (h)

Figure 4

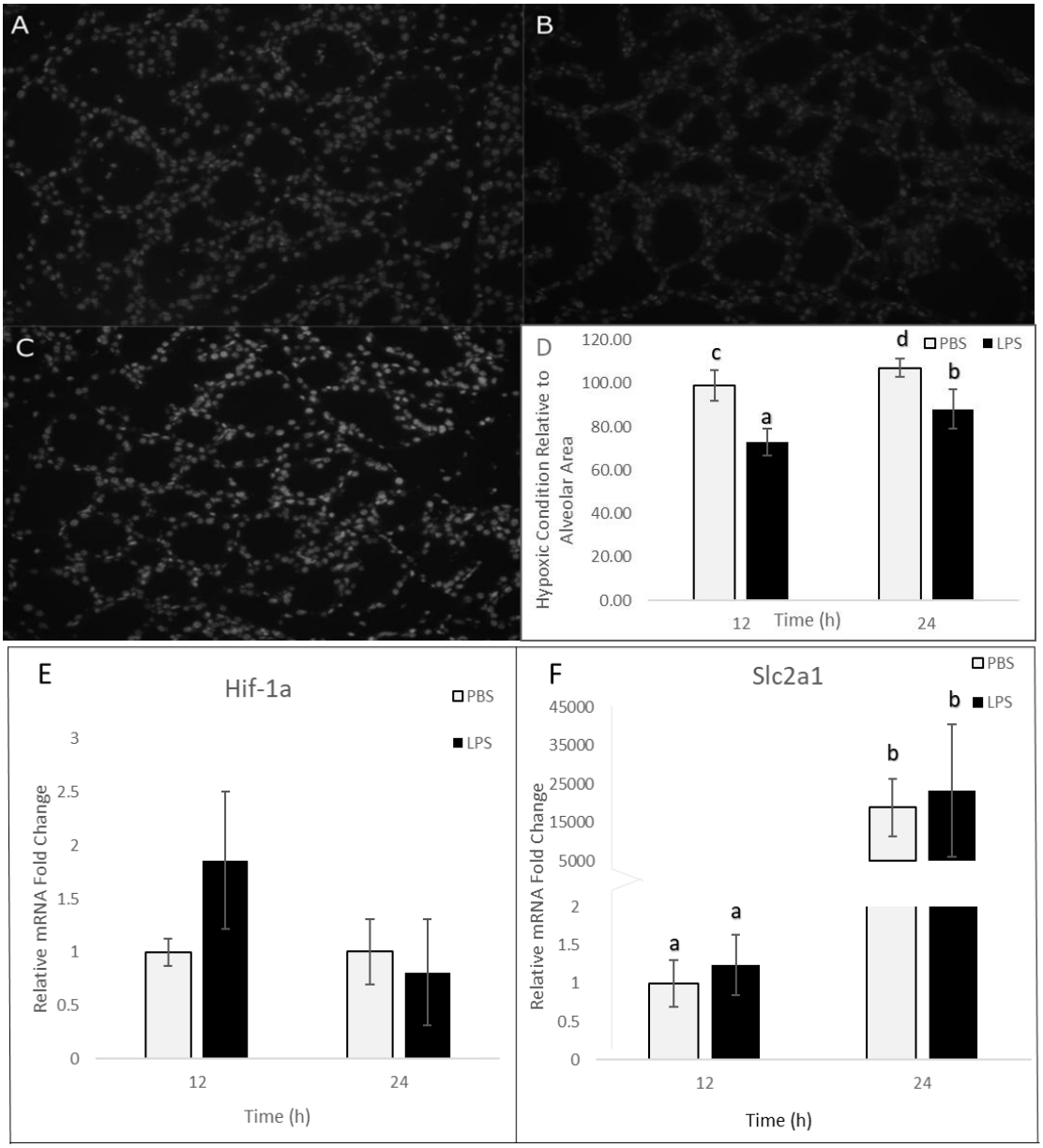
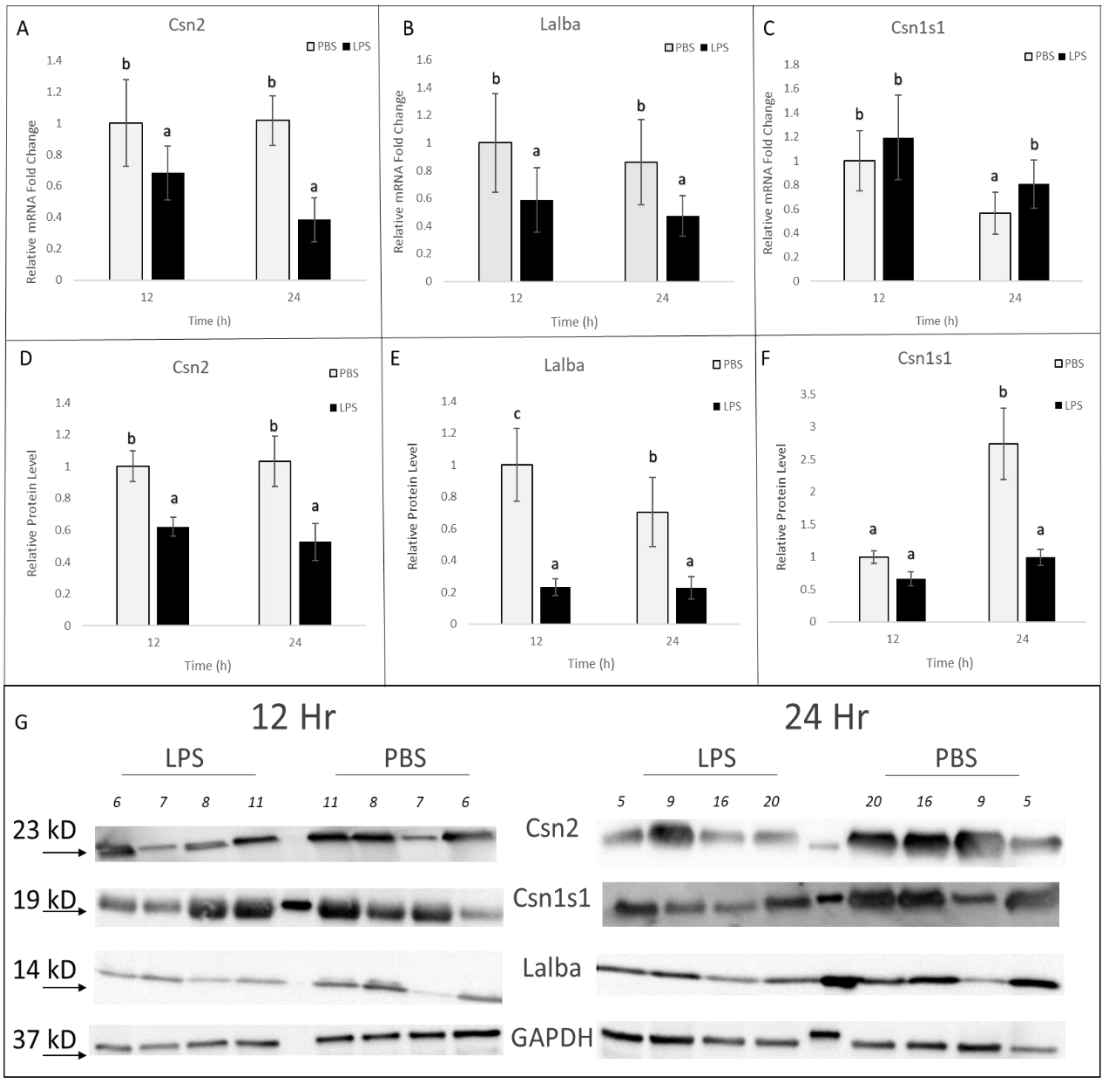

A
B
C
D
Hypoxic Condition Relative to Alveolar Area
120.00
100.00
80.00
60.00
40.00
20.00
0.00
c
a
d
b
PBS
LPS
12
Time (h)
24
E
Hif-1a
PBS
LPS
Relative mRNA Fold Change
3
2.5
2
1.5
1
0.5
0
12
24
Time (h)
F
Slc2a1
PBS
LPS
b
b
a
a
45000
35000
25000
15000
5000
2
1.5
1
0.5
0
Relative mRNA Fold Change
12
24
Time (h)

Figure 5


A
Csn2
PBS LPS
Relative mRNA Fold Change
Time (h)
B
Lalba
C
Csn1s1
D
Csn2
Relative Protein Level
E
Lalba
F
Csn1s1
G
12 Hr
24 Hr
LPS
PBS
23 kD
19 kD
14 kD
37 kD
Csn2
Csn1s1
Lalba
GAPDH

Chapter 3: General Discussion

Our study presents evidence that LPS challenge of the mammary gland induces programmed cell death in the early stage of mastitis. LPS challenge increases *Bax* gene expression and caspase 3 cleavage at 12 h in addition to raising oxidative stress and lowering the antioxidative capacity of the mammary gland. Other LPS IMI studies have also demonstrated similar trends of these individual markers, thus corroborating our findings (Habelhah *et al*., 2016; Zowalaty *et al*., 2018; Jeong *et al*., 2017). However, more information is needed to determine the cellular stimulator of LPS-induced apoptosis. Future research is required to compare the ER-stress in relation to mitochondrial dysfunction in LPS-stimulated mastitis. The ER crosstalks with the mitochondria of a cell through soluble factors, such as calcium, and protein:protein interactions between the organelles to coordinate anti-apoptotic or pro-apoptotic signaling among other functions (Malthotra *et al*., 2011; van Vliet *et al*., 2018; Senft *et al*., 2015). In addition, the use of Bax inhibitors in cancer therapies is an emerging field (Barclay *et al*., 2015; Li *et al*., 2017; Niu *et al*., 2017), and these compounds could be utilized in future research for therapeutics supporting mastitis recovery.

Our data show that while Nrf2-ARE genes *xCt* and *Nqo1* are upregulated, gene expression of *Nrf2* is not induced by LPS treatment. The possible reason for this discrepancy is that Nrf2 is primarily regulated at its translocation to the nucleus following dissociation from KEAP1. This research provides preliminary data to investigate the effects of antioxidative compounds or agonists that improve Nrf2-ARE signaling, such as polyphenolic compounds and tBHQ (Wang *et al*., 2019; Hu *et al*., 2018; Su *et al*., 2018;

Jin *et al*., 2016; Song *et al*., 2015) on milk protein synthesis and changes in cell death and stress during mastitis.

In this study, we observe a reduction in mRNA and protein expression of *Csn2* and *Lalba* by LPS challenge at both 12 and 24 h, but *Csn1s1* mRNA is increased at 24 h and its protein level is inhibited only at 24 h. These differential effects remain unclear, but this is evidence that alpha-S1 casein is a protein of interest in immune function. Vordenbäumen *et al.* (2016) have shown that alpha-S1 casein stimulates human monocyte differentiation to macrophages, but the impact of alpha-S1 casein expression *in vivo* remains unknown. Outside of this function, the control mechanisms of the differential expression of milk proteins remain unknown at the gene and protein levels. The protein abundance of alpha-S1 casein, unlike its mRNA, significantly decreases in the mammary gland at 24 h after LPS treatment, indicating an inhibition at the post-transcriptional levels.

One way to study the potential mechanism of the differential regulation of milk protein gene expression by LPS would be through chromatin immunoprecipitation assays to compare if there are unique interactions between NF-kB and the gene promotor of *Csn1s1* that are not occurring in neither the *Csn2* nor *Lalba* genes. In addition, future research of the impact mastitis has on *Csn2* and *Lalba* should elucidate how the gene and protein expression of *Csn2* and *Lalba* are repressed by the signaling of inflammation, oxidative stress, and programmed cell death pathways. Particularly, the interaction of pro-inflammatory NF-kB, antioxidative factors FOXO and Nrf2, cell survival factor AP-1, and p53 apoptosis transcription factor with the *Csn2* and *Lalba* promoters must be thoroughly examined through real time in mastitis (Zhang *et al*., 2018; Gào *et al*., 2017;

Qin *et al*., 2016; Kunnumakkara *et al*., 2018). The *Csn2* gene promoter has already been thoroughly examined for potential binding sites for most of these transcription factors, but they have not been investigated in mastitis (Malewski *et al*., 1995).

Another interesting study would be to determine if alpha-S1 casein is processed post-translationally in a manner that is not seen in other milk proteins like *Csn2* and *Lalba*. Saenger *et al*. (2019) found that unphosphorylated, but not phosphorylated *Csn1s1*, stimulated the inflammatory response. Lactation stage, parity, and genetic variation of cattle all contribute to different levels of milk protein phosphorylation throughout the lactation cycle of an individual animal, thus differential phosphorylation may result from mastitis too, which requires future research to confirm (Fang *et al*., 2017). There is also evidence of two different pathways of casein phosphorylation for low and high phosphorylated residues, thus the impact of mastitis on these pathways warrants investigation (Fang *et al*., 2016). Casein kinase 1 has been shown to be a negative regulator of FOXO, which promotes apoptosis due to ROS (Farhan *et al*., 2017). Casein kinase 2 has also shown differential activity as it is upregulated in hypoxic environments in a HIF-1α induced manner (Mottet *et al*., 2005).

Phosphorylation and post-translational proteolysis are two of the main controls for post-transcriptional regulation of milk protein expression. Proteolytic digestion of casein proteins can enhance their bioactivity and has also been shown to have immune impacts. α-S1 casein protein digests presented anti-hypertensive properties while β-casein peptides produce agonists of μ-opioid receptors which activate the immune response (Saito *et al*., 2003). The expression of such casein derivatives under inflammatory conditions is unclear, but pH, which increases in mastitis, has been shown to alter milk protein

structures and can dictate the activities of the proteases which act on the milk proteins (Gan *et al*., 2019). Another factor to consider is that bacteria secrete their own proteases, thus cleavage can vary from one infectious strain to another and may be a mechanism to evade the immune system (Gan *et al*., 2019).

A way to observe *Csn1s1* function in the immune system would be to compare the effects of mastitis on normal mice and *Csn1s1-/-* knockout mice. In such study, the effects of LPS intramammary infusion would be examined in relation to the inflammatory signaling and immune cell recruitment to see if less immune cells of different types are recruited in mammary glands of *Csn1s1-/-* knockout mice. *Csn2-/-* knockouts have been previously documented and showed that casein micelles were smaller in *Csn2-/-* knockout mice, and while the milk was not as nutritious as the wild-type, there was no difference in disease susceptibility (Kumar et al., 1994).

Our study demonstrates that the IMI of LPS enhances the oxygen tension of the mammary gland at 12 and 24 h as LPS glands present less localized hypoxia than in PBS treated glands. This was opposite to our original thoughts. In infections, increased HIF-1α is commonly observed in addition to higher glycolytic enzyme and VEGF gene expression (Ge *et al*., 2019). Thus, we originally hypothesized that this response is due to decreased oxygen tension resulting from the massive recruitment of immune cells. Our observation in this study contrasts with this hypothesis, which may be due to increasing blood supply during inflammation after 24 h or decreasing oxygen utilization due to a metabolic shift to an anaerobic metabolism.

While the oxygen tension is increased by LPS challenge in our study, the mRNA expression of HIF-1α and GLUT1, two hypoxia markers, is not increased. This can be

explained by 1) LPS does not induce hypoxia in the mammary gland and 2) HIF-1α is mainly regulated at its degradation and translocation. The treatment of smooth muscle cells with TNF-α also presented a failure to induce HIF-1α transcriptional activity in the inflammation-dependent nonhypoxic pathway (Richard *et al*., 2000; Tsapournioti *et al*., 2013). HIF-1α has been shown to activate AMPK, which inhibits biosynthetic pathways like the mTOR pathway (Bohensky *et al*., 2010), thus HIF-1α potentially may not participate in the repression of milk protein synthesis in LPS-induced mastitis. While it may not support milk synthesis, the promotion of hypoxia signaling in the face of inflammation can support the outcome of damage and cell death incurred by mastitis via metabolic reprograming and angiogenesis. Many studies have demonstrated that increasing HIF-1α expression increases the survival of cells and improves the prognosis of inflammatory conditions by using indirect agonists, such as PHD inhibitor GSK1278863 (Wigerup *et al*., 2016; Holdstock *et al*., 2016; Maxwell *et al*., 2016). HIF-1α activity can also be promoted by impairing its interaction with the repressive vHL (Xue *et al*., 2016). Investigation of effects of HIF-1α agonism or antagonism on the outcome of mastitis may be worthwhile to demonstrate the therapeutic potential of HIF-1α in future mastitis treatment research.

In this study, although we use a unilateral design to reduce individual differences between the treatment and control, large individual variances are still seen between animals in the expression of many genes. The expression of genes involved in oxidative stress and apoptosis, such as *xCt* and *Casp9* and *Chop*, present large numerical differences between LPS treatment and control, but a statistical difference is not able to be declared for these observations due to large standard errors. The large expression

variances between animals could have been due to asymmetric mammary development between individuals, differences in litter size, and milking frequency of the sampled gland (Robichaux *et al*., 2015; Wasson, 2017; Knight & Peaker, 1982; Murney *et al*., 2014; Alex *et al*., 2015), and these reasons support our unilateral model to increase the power of the treatment.

However, one of the weaknesses of our unilateral design is the potential presence of systemic effect of LPS. LPS injected into one gland may eventually enter the bloodstream and reach the opposite mammary gland. The systemic effects of LPS are widely documented (De Matteis *et al*., 2017; Guo *et al*., 2019; Kan *et al*., 2019; Pawlowski *et al*., 2016), thus, we cannot rule out the possibility of the presence of LPS effects on the PBS-injected gland in this study. Nevertheless, our study clearly shows the differences between LPS- and PBS-injected glands, indicating that any LPS effects on the PBS-injected gland should be minimal compared with the LPS-injected gland.

Another weakness of our study is that no milk was collected from the animals. While it is possible to milk mice and receive a substantial amount of milk for analyses, we decided not to collect milk from the animals in this study because one of our major objectives of this study was to examine if mastitis causes localized hypoxia in the mammary gland. The palpation process in milking and milk-letdown could have interfered with the oxygen tension of the mammary gland before tissue harvest and could also cause damage to the mammary gland to jeopardize the study (Keen *et al*., 1980).

A third drawback of this study is that our observations were done in tissue samples which contained many types of cells. Unlike immunohistochemistry, RT-qPCR and western blot analysis data may not be accurately representative of MEC specific gene

and protein expression, respectively. For example, infiltrating immune cells entering the mammary gland express pro-inflammatory cytokines and HIF-1α at greater amounts than most cells and could dilute the total expression of genes and proteins expressed by MECs (Zinkernagel *et al*., 2007). Thus, future studies should use laser capture microdissection in tissues to target specific cell populations or study specific cell effects *in vitro* and *in vivo*. The methods of cell isolation are just entering the field of dairy science and present a great opportunity to understand the cellular and molecular complexity that culminates in mastitis (Sridhara *et al*., 2017; Bach *et al*., 2017; Pal *et al*., 2017; Giraddi *et al*., 2018).

Bibliography

1. Abdelli, A., D. Raboisson, R. Kaidi, B. Ibrahim, A. Kalem, and M.Iguer-Ouada. Elevated non-esterified fatty acid and beta-hydroxybutyrate in transition dairy cows and their association with reproductive performance and disorders: A meta-analysis. Theriogenology (2017), 93; 99–104
2. Abdul Awal M, Matsumoto M, Toyoshima Y, Nishinakagawa H. Ultrastructural and morphometrical studies on the endothelial cells of arteries supplying the abdominoinguinal mammary gland of rats during the reproductive cycle. Journal of Veterinary Medical Science (1996), 58; 29-34
3. Abe T, Tohgi H, Isobe C, Murata T, Sato C. Remarkableincrease in the concentration of 8-hydroxyguanosine in cere-brospinal fluid from patients with Alzheimer's disease. Journal of Neuroscience Research (2002), 70(3); 447–50.
4. Aditya S, Humer E, Pourazad P, Khiaosaard R., Zebel, Q. Metabolic and stress responses in dairy cows fed a concentrate-rich diet and submitted to intramammary lipopolysaccharide challenge. Animal (2018), 12(4), 741-749.
5. Akers RM. Lactation and the Mammary Gland by (2002-07-02)
6. Akers RM. Major advances associated with hormone and growth factor regulation of mammary growth and lactation in dairy cows. Journal of Dairy Science (2006), 89(4); 1222–34.
7. Alam J, and Cook J. How Many Transcriptio n Factors Does It Take to Turn On the Heme Oxygenase-1 Gene? American Journal of Respiratory Cell and Molecular Biology (2006), 36(2); 166-174
8. Alex A, Collier J, Hadsell D, Collier R. Milk yield differences between 1× and 4× milking are associated with changes in mammary mitochondrial number and milk protein gene expression, but not mammary cell apoptosis or SOCS gene expression. Journal of Dairy Science (2015), 98(7); 4439-4448
9. Allision RD, Laven RA. Effect of vitamin E supplementation on the health and fertility of dairy cows: a review. Vet Record (2000), 147(25); 703-708
10. Alonzo, F. CCR5 is a receptor for Staphylococcus aureus leukotoxin ED. Nature (2012), 493; 51–55
11. Appuhamy J, Nayananjalie W, England E, Gerrard D, Akers R, Hanigan M. Effects of AMP-activated protein kinase (AMPK) signaling and essential amino acids on mammalian target of rapamycin (mTOR) signaling and protein synthesis rates in mammary cells. Journal of Dairy Science (2014), 97(1); 419-429.
12. Aruoma OI. Free radicals, oxidative stress, and antioxidants in human health and disease. Journal of theAmerican Oil Chemistry Society (1998), 75(2); 199–212.
13. Atwood CS, Hovey RC, Glover JP, Chepko G, Ginsburg E, Robison Jr WG, Vonderhaar BK. Progesterone induces side-branching of the ductal epithelium in the mammary glands of peripubertal mice. Journal of Endocrinology (2000), 167; 39–52
14. Bach K, Pensa S, Grzelak M, Hadfield J, Adams DJ, Marioni JC, Khaled WT. Differentiation dynamics of mammary epithelial cells revealed by single-cell RNA sequencing. Nature Communications (2017), 8, 2128.

15. Bagri DK, Pandey RK, Bagri GK, Kumari R, Khatik SK, Khatik P, Bagdi DL. mpact of subclinical mastitis on milk production in lactating cows in Varanasi. Journal of Entomolgy and Zoology Studies (2018), 6(5); 806-808
16. Ballard O, Morrow AL. Human Milk Composition: Nutrients and Bioactive Factors. Pediatric Clinics (2013), 60(1); 49-74
17. Barclay LA, Wales TE, Garner TP, Wachter F, Lee S, Guerra RM, Stewart ML, Braun CR, Bird GH, Gavathiotis E, Engen JR, Walensky LD. Inhibition of Pro-Apoptotic BAX by a Noncanonical Interaction Mechanism. Molecular Cell (2015), 57(5); 873-886.
18. Bang H.Y, Park SA, Saeidi S, Na HK, Surh, YJ. Docosahexaenoic Acid Induces Expression of Heme Oxygenase-1 and NAD(P)H:quinone Oxidoreductase through Activation of Nrf2 in Human Mammary Epithelial Cells. Molecules (2017), 22; 969
19. Barja G. The flux of free radical attack through mitochondrialDNA is related to aging rate. Aging (Milano) (2000), 12(5); 342–55.
20. Bast A. Oxidative stress and calcium homeostasis. In: Halliwell B, Aruoma OI, editors. DNA and free radicals. London: Ellis Horwood; (1993). 95–108
21. Baumgartner S, Mensink RP, Haenen GR, Bast A, Binder CJ, Bekers O, Husche C, Lütjohann D, Plat J. The effects of vitamin E or lipoic acid supplementation on oxyphytosterols in subjects with elevated oxidative stress: a randomized trial. Scientific Reports (2017), 7; 15288.
22. Beales PE, Elliott RB, Flohe S, Hill JP, Kolb H, Pozzilli P. A multi-centre, blinded international trial of the effect of A(1) and A(2) beta-casein variants on diabetes incidence in two rodent models of spontaneous type I diabetes. Diabetologia (2002), 45: 1240–1246.
23. Beaudry KL, Parsons CLM, Ellis SE, Akers RM. Localization and quantitation of macrophages, mast cells, and eosinophils in the developing bovine mammary gland. Journal of Dairy Science (2015), 99; 796-804.
24. Betts CB, Pennock ND, Caruso BP, Ruffell B, Borges VF, Schedin P. Mucosal Immunity in the Female Murine Mammary Gland. The Journal of Immunology (2018), 201(2); 734-746
25. Bhat AH, Dar KB, Anees S, Zargar MF, Masood A, Sofi MA, Ganie SA. Oxidative stress, mitochondrial dysfunction and neurodegenerative diseases; a mechanistic insight. Biomedicine & Pharmacotherapy (2015), 74; 101-110
26. Bhattarai D, Worku T, Dad R, Rehman ZU, Gong X, Zhang S. Mechanism of pattern recognition receptors (PRRs) and host pathogen interplay in bovine mastitis. Microbial Pathogenesis (2018), 120; 64-70
27. Bielski BHJ, Cabelli DE. Superoxide and hydroxyl radicalchemistry in aqueous solution. Active Oxygen in Chemistry (1996), 66–104
28. Bielski BHJ, Cabelli BH, Arudi RL, Ross AB. Reactivity ofRO2/O2. Radicals in aqueous solution. Journal of Physical Chemistry Ref Data (1985), 14:1041–100.
29. Biet J, Poole CA, Stelwagen K, Margerison JK, Singh K. Primary cilia distribution and orientation during involution of the bovine mammary gland. Journal of Dairy Science. (2016), 99(5); 3966-3978.

30. Bijl E, van Valenberg, Huppertz T, van Hooijdonk A, Bovenhuis H. Phosphorylation of αS1-casein is regulated by different genes Journal of Dairy Science (2014), 97(11); 7240 - 724
31. Bionaz M, Periasamy K, Rodriguez-Zas SL, Everts RE, Lewin HA, Hurley WL. Old and New Stories: Revelations from Functional Analysis of the Bovine Mammary Transcriptome during the Lactation Cycle. PLoS ONE (2012), 7(3): e33268.
32. Biswal S, Rizwan H, Pal S, Sabnam S, Parida P & Pal A. Oxidative stress, antioxidant capacity, biomolecule damage, and inflammation symptoms of sickle cell disease in children, Hematology (2019), 24(1); 1-9
33. Bohensky J, Leshinsky S, Srinivas V, Shapiro I. Chondrocyte autophagy is stimulated by HIF-1 dependent AMPK activation and mTOR suppression. Pediatric Nephrology (2010), 25(4); 633-642
34. Boscos C, Stefanakis A, Alexopoulos C, Samartzi F. Prevalence of subclinical mastitis and influence of breed, parity, stage of lactation and mammary bacteriological status on Coulter Counter Counts and California Mastitis Test in the milk of Saanen and autochthonous Greek goats. Small Ruminant Research (1996), 21(2); 139-147
35. Breyne K, De Vliegher S, De Visscher A, Piepers S, Meyer E. Technical note: A pilot study using a mouse mastitis model to study differences between bovine associated coagulase-negative staphylococci. Journal of Dairy Science (2015), 98(2); 1090-1100
36. Brun del Re I, Wu H. Arterial Blood Sampling Changes the Steady State of Glucose in Rats During FDG-PET. J Nuclear Medicine (2010), 51(2); 1745.
37. Buonfiglio D, Ramos-Lobo A, Silveira M, Furigo I, Hennighausen L, Frazão R, Donato Jr. J. Neuronal STAT5 signaling is required for maintaining lactation but not for postpartum maternal behaviors in mice. Hormones and Behavior (2015), 71; 60-68.
38. Burgos S, Cant J. IGF-1 stimulates protein synthesis by enhanced signaling through mTORC1 in bovine mammary epithelial cells. Domestic Animal Endocrinology (2010), 38(4); 211-221.
39. Burgos S, Dai M, Cant J. Nutrient availability and lactogenic hormones regulate mammary protein synthesis through the mammalian target of rapamycin signaling pathway. Journal of Dairy Science (2010), 93(1); 153–61.
40. Burns J, Yokota T, Ashihara H, Lean MEJ, Crozier A; Plant Foods and Herbal Sources of Resveratrol; Journal of Agricultural Food Chemistry (2002), 50; 3337−3340
41. Butterfield DA, Koppal T, Howard B, Subramaniam R, Hall N,Hensley K. Structural and functional changes in proteins induced by free radical-mediated oxidative stress and protective action of the antioxidants N-tert-butyl-alpha-phenylnitrone and vitamin E. Ann N Y Academy of Science (1998), 854; 448–62.
42. Campo Verde Arboccó F, Andres Persia F, Belén Hapon M, Jahn G. Hypothyroidism decreases JAK/STAT signaling pathway in lactating rat mammary gland. Molecular and Cellular Endocrinology (2017), 450; 14-23, ISSN 0303-7207

43. Carr D., Lau R., Hnatykiw A.D. cIAP2 Is an Independent Signaling and Survival Factor during Mammary Lactational Involution and Tumorigenesis. Journal of Mammary Gland Biology and Neoplasia (2018), 23; 109
44. Carter ME, Brunet A. FOXO transcription factors. Current Biology (2008), 17(4).
45. Cerruti PA. Pro-oxidant states and tumor activation. Science (1985), 227; 375–81
46. Chandler RL. Experimental bacterial mastitis in the mouse. Journal of Medical Microbiology (1970), 3; 273–282
47. Chen F, Chen B, Guan W, Chen J, Lv Y, Qiao H, Wang C, Zhang Y (2017). Metabolic Transition of Milk Lactose Synthesis and Up-Regulation vy AKT1 in Sows from Late Pregnancy to Lactation. Cell Biochemistry and Biophysics (2017), 75; 131-138.
48. Chevion M, Berenshtein E, Stadtman ER. Human studies related to protein oxidation: protein carbonyl content as a marker of damage. Free Radical Research (2000), 33; S99–108.
49. Chew BP. Antioxidant Vitamins Affect Food Animal Immunity and Health. The Journal of Nutrition (1995), 125(6); 1804S–1808S
50. Choi KM, Barash I, Rhoads RE. Insulin and prolactin synergistically stimulate β-casein messenger ribonucleic acid translation by cytoplasmic polyadenylation. Mol Endocrinol. (2004), 18(7); 1670–86.
51. Chu EY, Hens J, Andl T, Kairo A, Yamaguchi TP, Brisken C, Glick A, Wysolmerski JJ, Millar SE. Canonical WNT signaling promotes mammary placode development and is essential for initiation of mammary gland morphogenesis. Development. (2004), 131; 4819–4829.
52. Ci X, Zhou J, Lv H, Yu Q, Peng L, and Hua S. Betulin exhibits anti-inflammatory activity in LPS-stimulated macrophages and endotoxin-shocked mice through an AMPK/AKT/Nrf2-dependent mechanism., Cell Death and Disease (2017), 8; e2798
53. Clarke SR, Andre G, Walsh EJ, Dufrêne YF, Foster TJ, Foster SJ. Iron-Regulated Surface Determinant Protein A Mediates Adhesion of Staphylococcus aureus to Human Corneocyte Envelope Proteins. Infection and Immunity (2009), 77(6); 2408-2416
54. Colbert DC, McGarry MP, O'Neill K, Lee NA, Lee JJ. Decreased size and survival of weanling mice in litters of IL-5-/-mice are a consequence of the IL-5 deficiency in nursing dams. Contemporary Topics in Laboratory Animal Science (2005), 44(3); 53-55
55. Cole AL, Muthukrishnan G, Chong C, Beavis A, Eade CR, Wood MP, Deichen MG, Cole AM. Host innate inflammatory factors and staphylococcal protein A influence the duration of human Staphylococcus aureus nasal carriage. Mucosal Immunology (2016), 9; 1537-1548
56. Copple IM, Lister A, Obeng AD, Kitteringham NR, Jenkins RE, Layfield R, Foster BJ, Goldring CE., Park BK. Physical and functional interaction of sequestosome 1 with Keap1 regulates the Keap1-Nrf2 cell defense pathway, Journal of Biological Chemistry (2010), 285; 16782–16788.

57. Côté-Gravel J, Malouin F. Symposium review: Features of Staphylococcus aureus mastitis pathogenesis that guide vaccine development strategies. Journal of Dairy Science (2019), 102(5); 4727-4740
58. Couldrey C, Moitra J, Vinson C, Anver M, Nagashima K, Green J. Adipose tissue: A vital in vivo role in mammary gland development but not differentiation. Developmental Dynamics. (2002), 223; 459-468.
59. Cowin P, Wysolmerski J. Molecular mechanisms guiding embryonic mammary gland development. Cold Spring Harbor Perspective Biology (2010), 2
60. Dean RT, Fu S, Stocker R, Davies MJ. Biochemistry and pathology of radical-mediated protein oxidation. Journal of Biochemistry (1997), 324; 1–18.
61. Dego OK, van Dijk JE, Nederbragt H (2002) Factors involved in the early pathogenesis of bovine Staphylococcus aureus mastitis with emphasis on bacterial adhesion and invasion. A review, Veterinary Quarterly (2002), 24(4); 181-198
62. De Matteis L, Bertoni G, Lombardelli R, Wellnitz O, Van Dorland HA, Vernay, MC, Bruckmaier RM. and Trevisi E. (2017), Acute phase response in lactating dairy cows during hyperinsulinemic hypoglycaemic and hyperinsulinemic euglycaemic clamps and after intramammary LPS challenge. Journal of Animal Physiology and Animal Nutrition (2017), 101; 511-520.
63. Devraj G, Beerlage C, Volkhard BB, Kempf AJ. Hypoxia and HIF-1 activation in bacterial infections. Microbes and Infections (2017), 19; 144-156
64. Doppler W, Geymayer S, Weirich HG. Synergistic and Antagonistic Interactions of Transcription Factors in the Regulation of Milk Protein Gene Expression. In: Mol J.A., Clegg R.A. (eds) Biology of the Mammary Gland. Advances in Experimental Medicine and Biology (2002), 480. Springer, Boston, MA
65. Dürr JW, Cue RI, Monardes HG, Moro-Méndez J, Wade KM. Milk losses associated with somatic cell counts per breed, parity and stage of lactation in Canadian dairy cattle. Livestock Science (2008), 117 (2–3); 225-232
66. Dzięgelewska Z, Gajewska M. Stromal-Epithelial Interactions during Mammary Gland Development, Stromal Cells - Structure, Function, and Therapeutic Implications. IntechOpen (2018)
67. Elliott RB, Harris D, Hill J, Bibby NJ, Wasmuth HE. Type I (insulin-dependent) diabetes mellitus and cow milk: casein variant consumption. Diabetologia (1999), 42(3); 292-296
68. Elo T, Lindfors P, Lan Q, Voutilainen M, Trela E, Ohlsson C, Huh S, Ornitz D, Poutanen M, Howard B, Mikkola M. Ectodysplasin target gene Fgf20 regulates mammary bud growth and ductal invasion and branching during puberty. Scientific Reports (2017), 7; 5049
69. Endo M, Mori M, Akira S, Gotoh T. C/EBP Homologous Protein (CHOP) Is Crucial for the Induction of Caspase-11 and the Pathogenesis of Lipopolysaccharide-Induced Inflammation. Journal of Immunology (2006), 176; 6245-6253
70. Eslami H, Batavani RA, Asr I-Rezaei S, & Hobbenaghi R. Changes of stress oxidative enzymes in rat mammary tissue, blood and milk after experimental mastitis induced by E. coli lipopolysaccharide. Veterinary research forum: an international quarterly journal (2015), 6(2); 131–136

71. Esper RM, Dame M, McClintock S, Holt PR, Dannenberg AJ, Wicha MS, Brenner DE. Leptin and Adiponectin modulate the self-renewal of normal human breast epithelial stem cells. Cancer Prevention Research (Philadelphia, PA) (2015), 8(12); 1174-1183.
72. Fan WJ, Li HP, Zhu HZ, Sui SP, Chen PG, Deng Y, Sui TM, Wang YY. NF-κB is involved in the LPS-mediated proliferation and apoptosis of MAC-T epithelial cells as part of the subacute ruminal acidosis response in cows. Biotechnology Letters (2016), 38 (11); 1839–1849
73. Fang Z, Bovenhuis H, Delacroix-Buchet A, Miranda G, Boichard D, Visker M, Martin P. Genetic and nongenetic factors contributing to differences in αS-casein phosphorylation isoforms and other major milk proteins. Journal of Dairy Science (2017) 100 (7), 5564-5577.
74. Fang Z, Visker M, Miranda G, Delacroix-Buchet A, Bovenhuis H, and Martin P. The relationships among bovine a-S casein phosphorylation isoforms suggests different phosphorylation pathways. Journal of Dairy Science (2016), 99; 8168–8177
75. Farhan M, Wang H, Gaur U, Little PJ, Xu J, Zheng W. FOXO Signaling Pathways as Therapeutic Targets in Cancer. International journal of biological sciences (2017), 13(7); 815–827.
76. Farrell, HM, Jimenez-Flores R, Bleck GT, Brown EM, Butler JE, Creamer LK, Hicks CL, Hollar CM, Ng-Kwai-Hang KF, Swaisgood HE. Nomenclature of the proteins of cows' milk–Sixth revision. Journal of Dairy Science (2004), 87; 1641–1674.
77. Fenton HJH. Oxidation of tartaric acid in the presence of iron. Journal of Chemistry in Societal Translations. (1894), 65;899–910.
78. Feurmann Y, Mabjeesh S, Shamay A. Leptin Affects Prolactin Action on Milk Proein and Fat Synthesis in the Bovine Mammary Gland. Journal of Dairy Science (2004), 87(9), 2941-2946.
79. Filomeni G, De Zio D, Cecconi F. Oxidative stress and autophagy: the clash between damage and metabolic needs. Cell Death and Differentiation (2015), 22; 377–388
80. Fitzpatrick SF, Gojkovic M, Macias D, Tegnebratt T, Li Lu L, Samén E, Rundqvist H, Johnson RS. Glycolytic Response to Inflammation Over Time: Role of Myeloid HIF-1 alpha. Frontiers in Physiology (2018), 9(1624); 1-7.
81. Fornetti, J., Flanders, K. C., Henson, P. M., Tan, A. C., Borges, V. F., & Schedin, P. Mammary epithelial cell phagocytosis downstream of TGF-β3 is characterized by adherens junction reorganization. Cell death and differentiation (2015), 23(2); 185-96.
82. Gajewska M, Motyl T, IGF-binding proteins mediate TGF-β1-induced apoptosis in bovine mammary epithelial BME-UV1 cells, Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology (2004), 139(1–3); 65-75, ISSN 1532-0456
83. Gallo-Oller G, Ordoñez R, Dotor J. A new background subtraction method for Western blot densitometry band quantification through image analysis software. Journal of Immunological Methods (2018), 457; 1-5

84. Gan J, Robinson RC, Wang J, Krishnakumar N, Manning CJ, Lor Y, German JB. Peptidomic profiling of human milk with LC-MS/MS reveals pH-specific proteolysis of milk proteins. Food chemistry (2018), 274; 766–774.
85. Gào X., & Schöttker B. Reduction-oxidation pathways involved in cancer development: a systematic review of literature reviews. Oncotarget (2017), 8(31); 51888–51906.
86. Gao X, Zhang Z, Li Y, Hu X, Shen P, Fu YH, Cao Y, Zhang N. Selenium Deficiency Deteriorate the Inflammation of S. aureus Infection via Regulating NF-κB and PPAR-γ in Mammary Gland of Mice. Biological Trace Element Research (2016), 172(1); 140–147
87. Ge X, Wang L, Li M, Xu N, Yu F, Yang F, Li R, Zhang F, Zhao B, and Du J. Vitamin D/VDR signaling inhibits LPS-induced IFNγ and IL-1β in Oral epithelia by regulating hypoxia-inducible factor-1α signaling pathway. Cell Communication and Signaling (2019) 17:18
88. Geymayer S, Doppler W. Activation of NF-κB p50/p65 is regulated in the developing mammary gland and inhibits STAT5-mediated β-casein gene expression. The FASEB Journal (2000), 14:9, 1159-1170
89. Giraddi RR, Chung CY, Heinz RE, Balcioglu O, Novotny M, Trejo CL, Dravis C, Hagos BM, Mehrabad EM, Rodewald LW, Hwang JY, Fan C, Lasken R, Varley KE, Perou CM, Wahl GM, Spike BT. Single-Cell Transcriptomes Distinguish Stem Cell State Changes and Lineage Specification Programs in Early Mammary Gland Development. Cell Reports (2018), 24; 1653–1666.
90. Glaser P. Genome sequence of Streptococcus agalactiae, a pathogen causing invasive neonatal disease; Molecular Microbiology (2002), 45(6); 1499–1513
91. Gogoi-Tiwari J, Williams V, Waryah CB, Costantino P, Al-Salami H, Mathavan S. Mammary Gland Pathology Subsequent to Acute Infection with Strong versus Weak Biofilm Forming Staphylococcus aureus Bovine Mastitis Isolates: A Pilot Study Using Non-Invasive Mouse Mastitis Model. PLoS ONE (2017), 12(1): e0170668.
92. Gogoi-Tiwari J, Williams V, Waryah CB, Eto KY, Tau M, Costantino P, Tiwari HK, Mukkur T. Comparative studies of the immunogenicity and protective potential of biofilm vs planktonic Staphylococcus aureus vaccine against bovine mastitis using non-invasive mouse mastitis as a model system. Biofouling (2015), 31(7); 543-554
93. Gracner D, Gilligan G, Garvey N, Moreira L, Harvey P, Tierney A, Zobel LR. Correlation between the milk vein internal diameter surface and milk yield in Simmental cows. Turkish Journal of Veterinary and Animal Sciences (2015), 39; 741-744
94. Greer SN, Metcalf JL, Wang Y, Ohh M. The updated biology of hypoxia-inducible factor. The EMBO Journal (2012), 31(11).
95. Gregor MF, Misch ES, Yang L, Hummasti S, Inouye KE, Lee AH, Bierie B, Hotamisligil GS. The Role of Adipocyte XBP1 in Metabolic Regulation during Lactation. Cell Reports (2013), 3(5); 1430-1439
96. Gross J, van Dorland H, Bruckmaier R, Schwarz F, Performance and metabolic profile of dairy cows during a lactational and deliberately induced negative

energy balance with subsequent realimentation, Journal of Dairy Science (2011), 94(4), 1820-1830, ISSN 0022-0302
97. Guimarães JLB, Brito MAVP, Lange CC2, Silva MR, Ribeiro JB, Mendonça LC, Mendonça JFM, Souza GN. Estimate of the economic impact of mastitis: A case study in a Holstein dairy herd under tropical conditions. Preventative Veterinary Medicine (2017), 142; 46-50.
98. Günther J, Czabanska A, Bauer I, Leigh JA, Holst O, Seyfert HM. Streptococcus uberis strains isolated from the bovine mammary gland evade immune recognition by mammary epithelial cells, but not of macrophages. Veterinary Research (2016), 47(13)
99. Guo W, Liu B, Hu G, Kan X, Li Y, Gong Q, Xu D, Ma H, Cao Y, Huang B, Fu S, Liu J. Vanillin protects the blood–milk barrier and inhibits the inflammatory response in LPS-induced mastitis in mice. Toxicology and Applied Pharmacology (2019) 365; 9-18
100. Gyang EO, Stevens JB, Olson WG, Tsitsamis SD, Usenik EA. Effects of selenium-vitamin E injection on bovine polymorphonucleated leukocytes phagocytosis and killing of Staphylococcus aureus. American Journal of Veterinary Research (1984), 45(1); 175-177
101. Gyorki DE, Asselin-Labat ML, van Rooijen N, Lindeman GJ, Visvader JE. Resident macrophages influence stem cell activity in the mammary gland. Breast Cancer Research (2009), 11(4); R62
102. Habelhah H, Zhang L, Xialikaer A, Blackwell K. TRAF2 protects mammary epithelial and cancer cells from endoplasmic reticulum stress-induced apoptosis. In: Proceedings of the 107th Annual Meeting of the American Association for Cancer Research; 2016 Apr 16-20; New Orleans, LA. Philadelphia (PA): AACR; Cancer Res 2016; 76 (14 Suppl): Abstract nr 3496
103. Haber F, Weiss J. The catalytic decomposition of hydrogen per-oxide by iron salts. Proceedings of the Royal Society (A) (1934), 147; 332–51.
104. Habib P, Dreymueller D, Rösing B, Botung H, Slowik A, Al AZ, Habib S, Hoffmann S, Beyer C. Estrogen serum concentration affects blood immune cell composition and polarization in human females under controlled ovarian stimulation, The Journal of Steroid Biochemistry and Molecular Biology (2018), 178; 340-347, ISSN 0960-0760
105. Hagiwara S, Mori K, Nagahata H. Predictors of fatal outcomes resulting from acute Escherichia coli mastitis in dairy cows, Journal of Veterinary Medical Science (2016), 78(5); 905-908
106. Halliwell B, Clement MV, Long LH. Hydrogen peroxide in thehuman body. FEBS Letters (2000), 486(1); 10–3.
107. Hampton MB, Kettle AJ, Winterbourn CC. Inside the neutrophil phagosome: oxidants, myeloperoxidase, and bacterial killing. Blood (1998), 92(9); 3007–17.
108. Han LQ, Zhou Z, Ma Y, Batistel F, Osorio JS, Loor JJ. Phosphorylation of nuclear factor erythroid 2-like 2 (NFE2L2) in mammary tissue of Holstein cows during the periparturient period is associated with mRNA abundance of antioxidant gene networks. Journal of Dairy Science (2018), 101(7); 6511-6522

109. Hanayama R, Nagata S: Impaired involution of mammary glands in the absence of milk fat globule EGF factor 8. ProcNatl Acad Sci USA 2005, 102:16886-16891.
110. Hayashi AA, Nones K, Roy NC, McNabb WC, Mackenzie DS, Pacheco D, McCoard S. Initiation and elongation steps of mRNA translation are involved in the increase in milk protein yield caused by growth hormone administration during lactation. Journal of Dairy Science (2009), 92; 1889–99.
111. Hayashi AA, Proud CG. The rapid activation of protein synthesis by growth hormone requires signaling through mTOR. American Journal of Physiology Endocrinology and Metabolism (2007), 292(6); E1647–55.
112. Hayes J.D., McMahon M, Chowdhry S, Dinkova-Kostova AT. Cancer chemoprevention mechanisms mediated through the Keap1-Nrf2 pathway. Antioxidant.Redox Signaling (2010), 13; 1713–1748.
113. He X, Wei Z, Zhou E, Chen L, Kou J, Wang J, Yang Z. Baicalein attenuates inflammatory responses by suppressing TLR4 mediated NF-κB and MAPK signaling pathways in LPS-induced mastitis in mice. International Immunopharmacology (2015), 28(1); 470-476
114. Hinthong W, Pumipuntu N, Santajit S, Kulpeanprasit S, Buranasinsup S, Sookrung N, Chaicumpa W, Aiumurai P, Indrawattana N. Detection and drug resistance profile of Escherichia coli from subclinical mastitis cows and water supply in dairy farms in Saraburi Province, Thailand. PeerJ (2017), 5; e3431
115. Hiraku Y, Kawanishi S, Ichinose T, Murata M. The role of iNOS-mediated DNA damage in infection- and asbestos-induced carcinogenesis. Annual New York Academy of Science (2010), 1203; 15–22
116. Ho S, Woodford K, Kukuljan S, Pal S. Comparative effects of A1 versus A2 beta-casein on gastrointestinal measures: A blinded randomised cross-over pilot study. European Journal of Clinical Nutrition (2014), 68; 994–1000.
117. Hogan JS, Weiss WP, Smith KL. Role of Vitamin E and Selenium in Host Defense Against Mastitis. Journal of Dairy Science (1993), 76(9); 2795-2803
118. Holdstock L, Meadowcroft A, Maier R, Johnson B, Jones D, Rastogi A, Zeig S, Lepore J, and Cobitz A. Four-Week Studies of Oral Hypoxia-Inducible Factor–Prolyl Hydroxylase Inhibitor GSK1278863 for Treatment of Anemia. JASN (2016), 27 (4) 1234-1244.
119. Holland JK, Hadrich JC, Wolf CA, Jason L. Economics of Measuring Costs Due to Mastitis-Related Milk Loss. Research in Agricultural and Applied Economics (2015); 2-18
120. Holm L, Poulsen N, Thomsen B, Panitz F, Larsen L, Le TTT, Gregersen V. Differential Gene Expression In Mammary Gland Epithelial Cell between Holstein Individuals with High or Low Alpha-Lactalbumin Milk Protein Content. Proceedings of the World Congress on Genetics Applied To Livestock Production (2018)
121. Horsburgh MJ, Aish JL, White I.J, Lithgow JK, Foster SJ, Shaw L. sigmaB modulates virulence determinant expression and stress resistance: Characterization of a functional rsbU strain derived from Staphylococcus aureus. 8325-4. Journal of Bacteriology (2002), 184; 5457–5467.

122. Hovey RC, Aimo, L. Diverse and Active Roles for Adipocytes During Mammary Gland Growth and Function. Journal Mammary Gland Biology and Neoplasia (2010), 15; 279-290.
123. Hu G, Hong D, Zhang T, Duan H, Wei P, Guo X, Mu X. Cynatratoside-C from Cynanchum atratum displays anti-inflammatory effect via suppressing TLR4 mediated NF-κB and MAPK signaling pathways in LPS-induced mastitis in mice. Chemico-Biological Interactions (2018), 279; 187-195
124. Hughes K, Watson C. The multifaceted role of STAT3 in Mammary Gland Involution and Breast Cancer. Int. Journal of Molecular Science (2018), 19(6); 1695
125. Hughes K, Wickenden JA, Allen JE, Watson CJ. Conditional deletion of Stat3 in mammary epithelium impairs the acute phase response and modulates immune cell numbers during post-lactational regression. The Journal of Pathology (2012), 227(1):106-117.
126. Hwan S, Bao Y, Vasilis L, Androutsopoulos P, Tsukamotoc C, Shin TS, Tsatsakis AM, Chunga G. Short Communication: Anti-inflammatory effects of soyasapogenol I-αa via downregulation of the MAPK signaling pathway in LPS-induced RAW 264.7 macrophages. Food and Chemical Toxicology (2018), 113; 211-217
127. Ingvartsen KL, Moyes KM. Factors contributing to immunosuppression in the dairy cow during the periparturient period. Japanese Journal of Veterinary Research (2015), 63(1); S15-S24
128. Inman CK, Li N, Shore P. Oct-1 counteracts autoinhibition of Runx2 DNA binding to form a novel Runx2/Oct-1 complex on the promoter of the mammary gland-specific gene β-casein. Molecular Cell Biology (2005), 25(8); 3182–93.
129. Inman JL, Robertson C, Mott JD, Bissell MJ. Mammary gland development: Cell fate specification, stem cells and the microenvironment. Development (2015),142; 1028-1042.
130. Itoh K, Chiba T, Takahashi S, Ishii T, Igarashi K, Katoh Y, Oyake T, Hayashi N, Satoh K, Hatayama I, Yamamoto M, Nabeshima Y. An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements, Biochemical Biophysical Research Communications 236 (1997) 313–322.
131. Jain A, Lamark T, Sjottem E, Larsen K.B., Awuh J.A., Overvatn A, McMahon M, Hayes J.D., Johansen T. p62/SQSTM1 is a target gene for transcription factor NRF2 and creates a positive feedback loop by inducing antioxidant response element-driven gene transcription, Journal of Biological Chemistry (2010), 285; 22576–22591.
132. Janzen JJ. Economic Losses Resulting from Mastitis. A Review. Journal of Dairy Science (1970), 53(9); 1151–1160
133. Jensen RG. Handbook of Milk Composition. Academic Press (1995)
134. Jeong W, Bae H, Lim W, Bazer FW, Song G, Adiponectin: A prosurvival and proproliferation signal that increases bovine mammary epithelial cell numbers and protects them from endoplasmic reticulum stress responses, Journal of Animal Science (2017), 95(12); 5278–5289

135. Jia R, Du J, Cao L, Li Y, Johnson O, Gu Z, Jeney G, Xu P, Yin G. Antioxidative, inflammatory and immune responses in hydrogen peroxide-induced liver injury of tilapia (GIFT, Oreochromis niloticus). Fish and Shellfish Immunology (2019), 84; 894-905
136. Jiang K, Ma X, Guo S, Zhang T, Zhao G, Wu H, Wang X, Deng G. Anti-inflammatory Effects of Rosmarinic Acid in Lipopolysaccharide-Induced Mastitis in Mice. Inflammation (2018) 41(2); 437-448
137. Jiang Q, Chen S, Ren W, Liu G, Yao K, Wu G, Yin Y. Escherichia coli aggravates endoplasmic reticulum stress and triggers CHOP-dependent apoptosis in weaned pigs. Amino Acids (2017), 49(12); 2073–2082
138. Jin T, Bokarewa M, Foster T, Mitchell J, Higgins J, Tarkowski A. Staphylococcus aureus Resists Human Defensins by Production of Staphylokinase, a Novel Bacterial Evasion Mechanism. The Journal of Immunology (2004), 172(2); 1169-1176
139. Jin X, Wang K, Liu L, Liu H, Zhao FQ, Liu JX. Nuclear factor-like factor 2-antioxidant response element signaling activation by tert-butylhydroquinone attenuates acute heat stress in bovine mammary epithelial cells Journal of Dairy Science (2016), 99(11); 9094-9103
140. Jones W, Yu-Lee L, Clift S, Brown T, Rosen J (1985). The Rat Casein Multigene Family: Fine Structure and Evolution of the Beta-Casein Gene. The Journal of Biological Chemistry 260(11); 7042-7050.
141. Kan X, Liu B, Guo W, Wei L, Lin Y, Guo Y, Gong Q, Li Y, Xu D, Cao Y, Huang B, Dong A, Ma H, Fu S, Liu J. Myricetin relieves LPS-induced mastitis by inhibiting inflammatory response and repairing the blood–milk barrier. Journal Cell Physiology (2019), 1– 11
142. Kanovasky JR. Singlet oxygen production by biological systems. Chemical and Biological Interactions (1989), 70(1–2); 1–28.
143. Kanofsky JR. Singlet oxygen production by lactoperoxidase. Journal of Biological Chemistry. (1983), 258(10); 5991–3.
144. Kuhn F, Lassing C, Range A, Mueller M, Hunziker T, Ziemiecki, Andres AC. Pmg-1 and Pmg-2 constitute a novel family of KAP genes differentially expressed during skin and mammary gland development. Mechanisms of Development (1999), 86(1-2); 193-196
145. Keen C, Lonnerdal B, Sloan M and Hurley L. Effects of Milking Procedure on Rat Milk Composition. Physiology & Behavior (1980), 24; 613-615.
146. Ki SH, Cho IJ, Choi DW, Kim SG. Glucocorticoid Receptor (GR)-Associated SMRT Binding to C/EBPβ TAD and Nrf2 Neh4/5: Role of SMRT Recruited to GR in GSTA2 Gene Repression. Molecular and Cellular Biology (2005), 25(10); 4150-4165
147. Kim MR., Park DW., Lee JH., Choi DS., Hwang K.J, Ryu H.S., Min C.K.; Progesterone-dependent release of transforming growth factor-beta1 from epithelial cells enhances the endometrial decidualization by turning on the Smad signalling in stromal cells, MHR: Basic science of reproductive medicine (2005), 11(11), 801–808

148. Kitt AJ, Leigh JA. The Auxotrophic Nature of Streptococcus uberis. In: Horaud T., Bouvet A., Leclercq R., de Montclos H., Sicard M. (eds) Streptococci and the Host. Advances in Experimental Medicine and Biology (1997), 418. Springer, Boston, MA
149. Klotz LO, Steinbrenner H. Cellular adaptation to xenobiotics: Interplay between xenosensors, reactive oxygen species and FOXO transcription factors. Redox Biology (2017), 13; 646-654
150. Knight C, Peaker M. Mammary Development in Mice: Effects of Hemihysterectomy in Pregnancy and of Litter Size Postpartum. Journal of Physiology (1982), 327; 17-27
151. Kolek O, Gajkowska B, Godlewski, MM, Motyl, T. Molecular mechanism of TGF-beta1-induced apoptosis in HC11 mouse mammary epithelial cells (MEC). Cellular and Molecular Biology (2001) 47; 197–208.
152. Komatsu M, Kurokawa H, Waguri H, Taguchi K, Kobayashi H, Ichimura Y, Sou Y.S., Ueno I, Sakamoto A, Tong K, Kim M, Nishito Y, Iemura S, Natsume T, Ueno T, Kominami E, Motohashi H, Tanaka K, Yamamoto M, The selective autophagy substrate p62 activates the stress responsive transcription factor Nrf2 through inactivation of Keap1, Nature Cell Biology (2010), 12; 213–223.
153. Kuhn NJ, White A. Milk glucose as an index of the intracellularglucose concentration of rat mammary gland. Biochemical Journal (1975), 152(1);153–5.
154. Kumar S, Clarke AR, Hooper ML. Milk composition and lactation of beta-casein-deficient mice. published correction appears in Proceedings from the National Academy of Science (1994), 91(13); 6138–6142.
155. Kunnumakkara AB, Sailo BL, Banik K, Harsha C, Prasad S, Gupta SC, Bharti AC, and Aggarwal BB. Chronic diseases, inflammation, and spices: how are they linked? Journal of Translational Medicine (2018), 16(14).
156. Küper C, Beck FX, Neuhofer W. Toll-like receptor 4 activates NF-κB and MAP kinase pathways to regulate expression of proinflammatory COX-2 in renal medullary collecting duct cells. American Journal of Physiology-Renal Physiology (2012), 302:1, F38-F46
157. Landskroner-Eiger S, Park J, Israel D, Pollard JW, Scherer PE. Morphogenesis of the developing mammary gland: Stage-dependent impact of adipocytes. Developmental Biology (2010), 344; 968-978
158. Lau A, Wang X.J., Zhao F., Villeneuve N.F., Wu T., Jiang T, Sun Z, White E, Zhang D.D., A noncanonical mechanism of Nrf2 activation by autophagy deficiency direct interaction between Keap1 and p62, Molecular Cell Biology (2010), 30; 3275–3285.
159. Laukka T, Mariani CJ, Ihantola T, Cao JZ, Hokkanen J, Kaelin Jr WG, Godley LA, and Koivunen P. Fumarate and Succinate Regulate Expression of Hypoxia-inducible Genes via TET Enzymes. Journal of Biological Chemistry (2016), 291, 4256-4265.
160. Lee B, Kwon E, Kim Y, Hee J, Wook KS, Jin S, Lee K, Won D, won Sohn KJ, Kima TH, Jid JD. 1α,25-Dihydroxyvitamin D3 upregulates HIF-1 and TREM-1 via mTOR signaling. Immunology Letters (2015), 163(1); 14-21

161. Lee P, Jiang B, Chin BY, V. Iyer N, Alam J, Semenza G, Choi A. Hypoxia-inducible Factor-1 Mediates Transcriptional Activation of the Heme Oxygenase-1 Gene in Response to Hypoxia. Journal of Biological Chemistry (1997), 272(9); 5375–5381
162. Leehy KA, Truong TH, Mauro LJ, Lange CA, Progesterone receptors (PR) mediate STAT actions: PR and prolactin receptor signaling crosstalk in breast cancer models, The Journal of Steroid Biochemistry and Molecular Biology (2018), 176; 88-93, ISSN 0960-0760
163. Leitner G, Chaffer M, Shamay A, Shapiro F, Merin U, Ezra E, Saran A, Silanikove N. Changes in Milk Composition as Affected by Subclinical Mastitis in Sheep. Journal of Dairy Science (2004), 87(1); 46-52.
164. Lemay DG, Neville MC, Rudolph MC, Pollard KS, German JB. Gene regulatory networks in lactation: identification of global principles using bioinformatics. BMC Systems Biology (2007) 1(1); 56.
165. Leonard SS, Xia C, Jiang BH, Stinefelt B, Klandorf H, Harris GK, Shi X. Resveratrol scavenges reactive oxygen species and effects radical-induced cellular responses. Biochemical and Biophysical Research Communications (2003) 309(4); 1017-1026
166. Li C, Yang F, Liu F, Li D, Yang T. NRF2/HO-1 activation via ERK pathway involved in the anti-neuroinflammatory effect of Astragaloside IV in LPS induced microglial cells. Neuroscience Letters (2018), 666 (14); 104-11
167. Li F, Wang W, Cao Y, Liang D, Zhang W, Zhang Z, Jiang H, Guo M, Zhang N. Inhibitory effects of astragalin on lipopolysaccharide-induced inflammatory response in mouse mammary epithelial cells. Journal of Surgical Research (2014); 192; 573-581
168. Li H, Wang Z, Lin X, Yan Z, Cao Q, Zhao M, Shi K. MEN1/Menin Regulates Milk Protein Synthesis through mTOR Signaling in Mammary Epithelial Cells. Scientific Reports (2017), 7(5479).
169. Li L, Zhao X. Characterization of the resistance class 1 integrons in Staphylococcus aureus isolates from milk of lactating dairy cattle in Northwestern China. BMC Veterinary Research (2018), 14(59); 1-7
170. Li R, Ding C, Zhang J, Xie M, Park D, Ding Y, Chen G, Zhang G, Gilbert-Ross M, Zhou W, Marcus AI, Sun SY, Chen ZG, Sica GL, Ramalingam SS, Magis AT, Fu H, Khuri FR, Curran WJ, Owonikoko TK, Shin DM, Zhou J, and Deng X. Modulation of Bax and mTOR for Cancer Therapeutics. Cancer Research (2017), 77(11); 3001-3012.
171. Lin Y, Duan X, Lv H, Yang Y, Liu Y, Gao X, Hou X. The effects of L-type amino acid transporter 1 on milk protein synthesis in mammary glands of dairy cows, Journal of Dairy Science (2018), 101(2); 1687-1696
172. Lind M, Hayes A, Caprnda M, Petrovic D, Rodrigo L, Kruzliak P, Zulli A. Inducible nitric oxide synthase: Good or bad? Biomedicine & Pharmacotherapy (2017), 93; 370-375
173. Liu Y, Zhao Y, Skerry B, Wang X, Colin-Cassin C, Radisky D. C, Kaestner K. H., Li Z. Foxa1 is essential for mammary duct formation. Genesis (2016), 54; 277-285.

174. Lund L. R., Rømer J., Thomasset N., Solberg H., Pyke C., Bissell M. J., Danø K., Werb Z. Two distinct phases of apoptosis in mammary gland involution: proteinase-independent and -dependent pathways. Development (Cambridge, England) (1996), 122(1), 181-93.
175. Ma XM, Blenis J. Molecular mechanisms of mTOR-mediated translational control. Nature Reviews Molecular Biology (2009); 10; 307–18.
176. Maddox JF, Aherne K.M, Reddy C C. Sordillo LM. Increased neutrophil adherence and adhesion molecule mRNA expression in endothelial cells during selenium deficiency. Journal of Leukocyte Biology (1999), 65; 658-664
177. Magro G, Brevini TAL, Maglie MD, Minozzi G, Scanziani E, Piccinin R. An explant of heifer mammary gland to study the immune response of the organ. Research in Veterinary Science (2017), 44-50
178. Mailleux AA, Overholtzer M, Schmelzle T, Bouillet P, Strasser A, Brugge JS. BIM regulates apoptosis during mammary ductal morphogenesis, and its absence reveals alternative cell death mechanisms. Developmental Cell (2007), 12(2); 221–34.
179. Mailleux AA, Spencer-Dene B, Dillon C, Ndiaye D, Savona-Baron C, Itoh N, Kato S, Dickson C, Thiery JP, Bellusci S. Role of FGF10/FGFR2b signaling during mammary gland development in the mouse embryo. Development (2002), 129(1); 53-60
180. Makarem M, Spike BT, Dravis C, Kannan N, Wahl GM, Eaves CJ. Stem cells and the developing mammary gland. Journal of Mammary Gland Biology and Neoplasia. (2013), 18(2); 209-219.
181. Malewski T, Zwierzchowski L. Computer-aided analysis of potential transcription-factor binding sites in the rabbit P-casein gene promoter. BioSystems (1995), 36; 109-l19.
182. Malhotra, J. D., & Kaufman, R. J. ER stress and its functional link to mitochondria: role in cell survival and death. Cold Spring Harbor perspectives in biology (2011); 3(9), a004424.
183. Maller O, Martinson H, Schedin P. Extracellular matrix composition reveals complex and dynamic stromal-epithelial interactions in the mammary gland. Journal of Mammary Gland Biology and Neoplasia (2010), 15; 301-318.
184. Mandal, PK, Seiler A, Perisic T, Kölle P, Canak AB, Förster H, Conrad M. System x(c)- and thioredoxin reductase 1 cooperatively rescue glutathione deficiency. The Journal of biological chemistry (2010), 285(29); 22244–22253
185. Manjarín R, Steibel J, Kirkwood R, Taylor N, Trottier N. Transcript abundance of hormone receptors, mammalian target of rapamycin pathway-related kinases, insulin-like growth factor I, and milk proteins in porcine mammary tissue. Journal of Animal Science (2012), 90(1); 221–30.
186. Marciniak A, Suwal S, Brisson G, Britten M, Pouliot Y, Doyen A. Studying a Chaperone-Like Effect of Beta-Casein on Pressure-Induced Aggregation of Beta-Lactoglobulin in the Presence of Alpha-Lactalbumin. Food Hydrocollods (2018), 84; 9-15.
187. Martinet W, de Meyer GR, Herman AG, Kockx MM. Reactive oxygen species induce RNA damage in human atherosclerosis. European Journal of Clinical Investigation (2004), 34(5); 323–7.

188. Martínez-Chacón G, Saarinen N, Mäkelä S. CC chemokine ligand 2 (CCL2) stimulates local aromatase expression in mammary adipose tissue via MAPK/ERK signaling pathway. The FASEB Journal (2019) 33(1); 580.4-580.4
189. Martinson HA, Jindal S, Durand-Rougely C, Borges VF, Schedin, P. (2015), Wound healing-like immune program facilitates postpartum mammary gland involution and tumor progression. International Journal of Cancer (2015), 136; 1803-1813.

190. Matsumoto, T., Nakamura E, Nakamura H, Hirota M, San Gabriel A, Nakamura K, Chotechuang N, Wu G, Uneyama H, and Torii K. 2013. Production of free glutamate in milk requires the leucine transporter LAT1. Am. J. Physiol. Cell Physiol. 305:C623–C631.
191. Matsunaga K, Tsugami Y, Kumai A, Suzuki T, Nishimura T, Kobayashi K. IL-1β directly inhibits milk lipid production in lactating mammary epithelial cells concurrently with enlargement of cytoplasmic lipid droplets. Experimental Cell Research (2018), 370 (2); 365-372

192. Matthews DG, D'Angelo J, Drelich J, Welsh JE. Adipose-specific Vdr deletion alters body fat and enhances mammary epithelial density. The Journal of Steroid Biochemistry and Molecular Biology (2016), 164; 299-308

193. Mavangira V, Sordillo LM. Role of lipid mediators in the regulation of oxidative stress and inflammatory responses in dairy cattle Research in Veterinary Science (2018), 116; 4-14
194. Maxwell P, Eckardt KU. HIF prolyl hydroxylase inhibitors for the treatment of renal anaemia and beyond. Nature Reviews Nephrology (2016), 12; 157–168.
195. McFadden T, Akers R, Capuco A. Relationship of Milk Proteins in Blood with Somatic Cell Count in Milk in Dairy Cows. Journal of Dairy Science (1987), 71; 826-834
196. McGuinness WA, Kobayashi SD, De Leo FR. Evasion of Neutrophil Killing by Staphylococcus aureus. Pathogens (2016), 5; 32.

197. Memon MA, Wang Y, Xu T, Ma N, Zhang H, Roy AC, Aabdin ZU. Lipopolysaccharide induces oxidative stress by triggering MAPK and Nrf2 signaling pathways in mammary glands of dairy cows fed a high-concentrate diet. Microbial Pathogenesis (2019), 128; 268-275
198. Menegazzi R, Decleva E, Dri P. Killing by neutrophil extracellular traps: fact or folklore? Blood (2012), 119; 1214-1216

199. Middleton JR. Staphylococcus aureus antigens and challenges in vaccine development, Expert Review of Vaccines (2008), 7(6); 805-815
200. Minuti A, Zhou Z, Graugnard DE, Rodriguez-Zas SL, Palladino AR, Cardoso FC, Trevisi E, Loor JJ. Acute mammary and liver transcriptome responses after an intramammary Escherichia coli lipopolysaccharide challenge in postpartal dairy cows. Physiological Reports (2015), 3(4); e12388.
201. Mir BA, Razak R, Ali A, Muzamil S, Mir MR, Khaliq T, Baba OK, Rehman MU, Bilal S, Hussain I, Rashid IM. Assessment of antioxidant profile in

subclinical and clinical mastitis in dairy cattle. Journal of Entomology and Zoology Studies (2017), 5(6); 1022-1025
202. Monks J. TGFβ as a potential mediator of progesterone action in the mammary gland of pregnancy. Journal of Mammary Gland Biology and Neoplasia (2007), 12(4); 249–57
203. Morad V, Abrahamsson A, Kjölhede P, Dabrosin C. Adipokines and vascular endothelial growth factor in normal human breast tissue in vivo—Correlations and attenuation by dietary flaxseed. Journal of Mammary Gland Biology and Neoplasia. (2016), 21; 69-76
204. Mottet D, Pyr Dit Ruys S, Demazy C, Raes M, Michiels C. Role for casein kinase 2 in the regulation of HIF-1 activity. International Journal of Cancer (2005), 117; 764–774
205. Murney R, Stelwagen K, Wheeler T, Margerison J, Singh K. The effects of milking frequency in early lactation on milk yield, mammary cell turnover, and secretory activity in grazing dairy cows. Journal of Dairy Science (2014), 98: 305–311
206. Namani A, Li Y, Wang XJ, Tang X. Modulation of NRF2 signaling pathway by nuclear receptors: Implications for cancer, Biochimica et Biophysica Acta: Molecular Cell Research (2014), 1843(9); 1875-1885, ISSN 0167-4889
207. Navas-Madroñal M, Vila L, Fité J, Martinez-Gonzalez J, Rodriguez C, Camacho M, Galán M. Targeting endoplasmic reticulum stress as a therapy to manage abdominal aortic aneurysm disease. Atherosclerosis (2018), 275; e53
208. Need EF, Atashgaran V, Ingman WV, Dasari P. Hormonal regulation of the immune microenvironment in the mammary gland. Journal of Mammary Gland Biology and Neoplasia (2014), 19(2); 229-239
209. Neville M, McFadden T, and Forsyth I. Hormonal Regulation of Mammary Differentiation and Milk Secretion. Journal of Mammary Gland Biology and Neoplasia (2002) 7(1).
210. Neville M, Monks J. The Cell Biology of the Lactating Epithelium. Reference Module in Biomedical Research: Encyclopedia of Reproduction Second Edition (2018), 2; 779-785
211. Nguyen AV, Pollard J: Transforming growth factor beta3 induces cell death during the first stage of mammary gland involution. Development (2000), 127; 3107-3118.
212. Nguyen T, Sherratt PJ, and Pickett CB. Regulatory Mechanisms Controlling Gene Expression Mediated by the Antioxidant Response Element. Annual Review of Pharmacology and Toxicology (2003), 43(1); 233-260
213. Nicoleta S, Rapeanu G. An Overview of Bovine Alpha-Lactalbumin Structure and Functionality. Annals of the University Dunarea des Jos of Galati, Fascicle VI-Food Technology (2010), 34(2)
214. Nishitoh H. CHOP is a multifunctional transcription factor in the ER stress response. The Journal of Biochemistry (2012), 151(3); 217–219
215. Niu X, Brahmbhatt H, Mergenthaler P, Zhang Z, Sang J, Daude M, Ehlert FGR, Diederich WE, Wong E, Zhu W, Pogmore J, Nandy JP, Satyanarayana M, Jimmidi RK, Arya P, Leber B, Lin J, Culmsee C, Yi J, Andrew DW. A Small-

Molecule Inhibitor of Bax and Bak Oligomerization Prevents Genotoxic Cell Death and Promotes Neuroprotection. Cell Chemical Biology (2017), 24; 493–50.
216. Notebaert S, Meyer E. Mouse models to study the pathogenesis and control of bovine mastitis. A review, Veterinary Quarterly (2006), 28(1); 2-13
217. Novick RP. Autoinduction and signal transduction in the regulation of staphylococcal virulence. Molecular Microbiology (2003), 48; 1429-1449.
218. Ogola H, Shitandi A, Nanua J. Effect of mastitis on raw milk compositional quality. Journal of Veterinary Science (2007), 8(3); 237-242
219. Ornitz DM, Itoh N. The fibroblast growth factor signaling pathway. Wiley interdisciplinary reviews. Developmental Biology (2015), 4(3); 215-266
220. Otto M. Staphylococcal Infections: Mechanisms of Biofilm Maturation and Detachment as Critical Determinants of Pathogenicity. Annual Review of Medicine (2013), 64(1); 175-188
221. Padberg S, Schumm-Draeger PM, Petzoldt R, Becker F, Federlin K. The significance of A1 and A2 antibodies against beta-casein in type-1 diabetes mellitus. Dtsch Med Wochenschr (1999), 124; 1518–1521.
222. Påhlman S, Lund LR, Jögi A. Differential HIF-1α and HIF-2α Expression in Mammary Epithelial Cells during Fat Pad Invasion, Lactation, and Involution. PLoS ONE (2015), 10(5): e0125771.
223. Pal B, Chen Y, Vaillant F, Jamieson P, Gordon L, Rios AC, Wilcox S, Fu N, Liu KH, Jackling FC, Davis MJ, Lindeman GL, Smyth GK & Visvader JE. Construction of developmental lineage relationships in the mouse mammary gland by single-cell RNA profiling. Nature Communications (2017), 8; 1627.
224. Park YK , Fox LK, Hancock DD, McMahan W, Park YH. Prevalence and antibiotic resistance of mastitis pathogens isolated from dairy herds transitioning to organic management; Journal Veterinary Science (2012), 13(1); 103-105
225. Pavlovich AL, Manivannan S, Nelson CM. Adipose stroma induces branching morphogenesis of engineered epithelial tubules. Tissue Engineering Part A. (2010), 16(12); 3719-2376.
226. Pawlowski K, Leroux C, Faulconnier Y, Boby C, de la Foye A, Durand D, and Pires J. Liver transcriptome modifications by nutrient restriction in early lactation Holstein cows challenged with intramammary lipopolysaccharide. Journal of Animal Science (2016), 94(5); 60–61
227. Peters MDP, Silveira IDB, Fischer V. Impact of subclinical and clinical mastitis on sensitivity to pain of dairy cows. Animal (2015), 9(12); 2024-2028
228. Pfitzner E, Jahne R, Wissler M, Stoecklin E, Groner B. p300/CREB-binding protein enhances the prolactin-mediated transcriptional induction through direct interaction with the transactivation domain of Stat5, but does not participate in the Stat5-mediated suppression of the glucocorticoid response. Molecular Endocrinology (1998), 12(10); 1582–93
229. Plafker KS, Nguyen L, Barneche M, Mirza S, Crawford D, Plafker SM. The ubiquitin-conjugating enzyme UbcM2 can regulate the stability and activity of the antioxidant transcription factor Nrf2, Journal Biological Chemistry (2010), 285; 23064–23074.

230. Prutz WA. Hypochlorous acid interactions with thiols, nucleo-tides, DNA, and other biological substrates. Archives of Biochemistry and Biophysics. (1996), 332 (1); 110–20.
231. Qian X, Zhao FQ. Current Major Advances in the Regulation of Milk Protein Gene Expression. Critical Reviews in Eukaryotic Gene Expression (2014), 24(4); 357–378
232. Qian X, Zhao FQ. Interactions of the ubiquitous octamer-binding transcription factor-1 with both the signal transducer and activator of transcription 5 and the glucocorticoid receptor mediate prolactin and glucocorticoid induced β-casein gene expression in mammary epithelial cells. International Journal of Biochemistry and Cell Biology (2013), 45(3); 724–35
233. Qin, S. and Hou, D. (2016), Multiple regulations of Keap1/Nrf2 system by dietary phytochemicals. Molecular Nutrition in Food Research (2016), 60; 1731-1755.
234. Quaglino A, Schere-Levy C, Romorini L, Meiss R, Kordon E. Mouse Mammary Tumors Display Activation Dependent on Leukemia Inhibitory Factor Signaling. Breast Cancer Research (2007), 9; 69
235. Ray PD, Huang BW, Tsuji Y. Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. Cellular Signaling (2012), 24(5); 981-990
236. Reichenstein M, Rauner G, Barash I. Conditional repression of STAT5 expression during lactation reveals its exclusive roles in mammary gland morphology, milk-protein gene expression, and neonate growth. Molecular Reproductive Development (2011), 78; 585-596.
237. Richard D, Berra E and Pouysségur J. Nonhypoxic Pathway Mediates the Induction of Hypoxia-inducible Factor 1α in Vascular Smooth Muscle Cells. The Journal of Biological Chemistry (2000), 275; 26765-26771
238. Richert M., Schwertfeger K., Ryder J., Anderson S. (2000). "An Atlas of Mouse Mammary Gland Development." Journal of Mammary Gland Biology and Neoplasia, 5(2); 227-241
239. Riskin A, Mond Y. Prolactin-Inducd Subcellular Targeting of GLUT1 Glucose Transporter in Living Mammary Epithelial Cells. Rambam Maimonides Medical Journal (2015), 6(4); e0038
240. Rius A, Appuhamy J, Cyriac J, Kirovski D, Becvar O, Escobar J, McGilliard M, Bequette B, Akers R, Hanigan M, Regulation of protein synthesis in mammary glands of lactating dairy cows by starch and amino acids, Journal of Dairy Science (2010), 93(7); 3114-3127, ISSN 0022-0302
241. Robichaux J, Hallett R, Fuseler J, Hassell J, & Ramsdell A. Mammary glands exhibit molecular laterality and undergo left–right asymmetric ductal epithelial growth in MMTV-cNeu mice. Oncogene (2015), 34, 2003–2010.
242. Ross D, Siegel D. Functions of NQO1 in Cellular Protection and CoQ10 Metabolism and its Potential Role as a Redox Sensitive Molecular Switch. Frontiers in physiology (2017), 8; 595.
243. Ryman VE, Packiriswamy N, Sordillo LM. Role of endothelial cells in bovine mammary gland health and disease. Animal Health Research Reviews (2015), 16(2); 135-149.

244. Ryter SW, Choi AMK. Targeting heme oxygenase-1 and carbon monoxide for therapeutic modulation of inflammation. Translational Research (2016), 167(1); 7-34
245. Saenger T, Vordenbäumen S, Genich S, Haidar S, Schulte M, Nienberg C, Bleck E, Schneider M, Jose J, Human αS1-casein induces IL-8 secretion by binding to the ecto-domain of the TLR4/MD2 receptor complex, Biochimica et Biophysica Acta (BBA) - General Subjects (2019), 1863(3); 632-643 ISSN 0304-4165
246. Saito, T.; Nakamura, T.; Kitazawa, H.; Kawai, Y.; Itoh, T. Isolation and structural analysis of antihypertensive peptides that exist naturally in Gouda cheese. Journal of Dairy Science (2000), 83; 1434–1440
247. Sakakura T. Mammary embryogenesis. In M. C. Neville and C. W. Daniel (eds.), The Mammary Gland: Development, Regulation, and Function, Plenum Press (1987), New York, 37–65
248. Sandahl M., Hunter D. M., Strunk K. E., Earp H. S., & Cook R. S. Epithelial cell-directed efferocytosis in the post-partum mammary gland is necessary for tissue homeostasis and future lactation. BMC developmental biology (2010), 10; 122
249. Santos G, Bottino MP, Santos APC, Simões LMS, Souza JC, Ferreira MBD, da Silveira JC, Ávila ACFCM, Bride A, Sales JNS. Subclinical mastitis interferes with ovulation, oocyte and granulosa cell quality in dairy cows. Theriogenology (2018), 119; 214-219
250. Sargeant T, Lloyd-Lewis B, Resemann H, Ramos-Montoya A, Skepper J, Watson C. Stat3 controls cell death during mammary gland involution by regulating uptake of milk fat globules and lysosomal membrane permeabilization. Nature Cell Biology (2014), 16; 1057
251. Schingoethe DJ, Casper DP, Yang C, Illg DJ, Sommerfeldt JL, Mueller CR. Lactational Response to Soybean Meal, Heated Soybean Meal, and Extruded Soybeans with Ruminally Protected Methionine. Journal of Dairy Science (1988), 71(1); 173-180.
252. Schuler F, Baumgartner F, Klepsch V, Chamson M, Möller-Holzner E, Watson C, Oh S, Hennighausen L, Tymoszuk P, Doppler W, Villunger A. The BH3-only protein BIM contributes to late-stage involution in the mouse mammary gland. Cell Death and Differentiation (2016), 23; 41-51.
253. Schweikl H, Gallorini ML, Pöschl G, Vera, Urmanna V, Petzel C, Bolay C, Hiller KA, Cataldi A, Buchall W. Functions of transcription factors NF-κB and Nrf2 in the inhibition of LPS-stimulated cytokine release by the resin monomer HEMA. Dental Materials (2018), 34(11); 1661-1678
254. Seegers H, Fourichon C, Beaudeau F. Production effects related to mastitis and mastitis economics in dairy cattle herds. Veterinary Research, BioMed Central (2003), 34(5); 475-91.
255. Sendi P, Proctor RA. Staphylococcus aureus as an intracellular pathogen: the role of smallcolony variants. Trends in Microbiology (2009), 17(2); 54-58
256. Senft D, Ronai ZA. UPR, autophagy, and mitochondria crosstalk underlies the ER stress response. Trends in Biochemical Sciences (2015),40(3); 141-148.

257. Serruto D, Rappuoli R, Scarselli M, Gros P, van Strijp JAG. Molecular mechanisms of complement evasion: learning from staphylococci and meningococci. Nature Reviews Microbiology (2010), 8; 393–399

258. Shaheen M, Tantary SM HA, Nabi SU. A Treatise on Bovine Mastitis: Disease and Disease Economics, Etiological Basis, Risk Factors, Impact on Human Health, Therapeutic Management, Prevention and Control Strategy. Journal of Advances in Dairy Research (2016), 4(1); 1-10

259. Shao Y, Zhao FQ. Emerging evidence of the physiological role of hypoxia in mammary development and lactation. Journal of Animal Science and Biotechnology (2014), (5); 9.

260. Shao Y, Wall E, McFadden T, Misra Y, Qian X, Blauwiekel R, Kerr D, Zhao FQ. Lactogenic Hormones Stimulate Expression of Lipogenic Genes but Not Glucose Transporters in Bovine Mammary Gland. Domestic Animal Endocrinology (2013), 44; 57-69.

261. Shao Y, Wellman TL, Lounsbury KM, and Zhao FQ. Differential regulation of GLUT1 and GLUT8 expression by hypoxia in mammary epithelial cells. American Journal of Physiology Regulation and Integrated Comparative Physiology (2014), 307; R237–R247

262. Shennan DB, Grant A, Ramsay RR, Burns C, Zammita VA. Characteristics of L-carnitine transport by lactating rat mammary tissue. Biochimica et Biophysica Acta (BBA) - Lipids and Lipid Metabolism (1998), 1393(1); 49-56

263. Shi H, Zhu JJ, Luo J, Cao W, Shi H, Yao D, Li J, Sun Y, Xu H,Yu K, Loor JJ. Genes regulating lipid and protein metabolism are highly expressed in mammary gland of lactating dairy goats. Functional & Integrative Genomics (2015), 15(3), 309–321

264. Silanikove N, Rauch-Cohen A, Shapiro F, Blum S, Arieli A, Leitner G. Lipopolysaccharide challenge of the mammary gland in bovine induced a transient glandular shift to anaerobic metabolism. Journal of Dairy Science (2011), 94(9); 4468-4475

265. Sinowatz F, Schams D, Kolle S, Plath A, Lincoln D, Waters M. Cellular localisation of GH receptor in the bovine mammary gland during mammogenesis, lactation and involution. Journal of Endocrinology (2000), 166(3); 503–10.

266. Smith C, Brew K. Isolation and Characteristics of Galactosyltransferase from Golgi Membranes of Lactating Sheep Mammary Glands. The Journal of Biological Chemistry (1977), 252(20); 7294-7299.

267. Smith KL, Harrison JH, Hancock DD, Todhunter DA, Conrad HR. Effect of Vitamin E and Selenium Supplementation on Incidence of Clinical Mastitis and Duration of Clinical Symptoms. Journal of Dairy Science (1984), 67(6); 1293-1300

268. Smith KL, Hogan JS, Weiss WP. Dietary vitamin E and selenium affect mastitis and milk quality. Journal of Animal Science (1997), 75(6); 1659–1665

269. Song X, Wang T, Zhang Z, Jiang H, Wang W, Cao Y, Zhang N. Leonurine Exerts Anti-Inflammatory Effect by Regulating Inflammatory Signaling Pathways and Cytokines in LPS-Induced Mouse Mastitis. Inflammation (2015), 38; 79.

270. Sordillo LM. Symposium review: Oxylipids and the regulation of bovine mammary inflammatory responses Journal of Dairy Science (2018), 101(6); 5629-5641, ISSN 0022-0302
271. Sprong H, Degroote S, Nilsson T, Kawakita M, Ishida N, van der Sluijs P, van Meer G. Association of the Golgi UDP-Galactose Transporter with UDP-Galactose: ceramide galactoslytransferase Allows UDP-Galactose Import in the Endoplasmic Reticulum. Mol. Biol. Cell. (2003), 14(8); 3482-93
272. Sridhara V, Liu T, Gritsenko MA, Sturtz LA, Kovatich AJ, Petyuk VA, Deyarmin B, McDermott JE, Shukla AK, Moore RJ, Monroe ME, Webb-Robertson BJM, Hooke JA, Fantacone-Campbell L, Kumar PKR, Kvecher L, Liu J, Kane J, Melley J, Somiari S, Iida J, Benz SC, Golovato J, Rabizadeh S, Soon-Shiong P, Smith RD, Mural RJ, Shriver CD, Hu H, and Rodland KD. Integrated proteogenomic analysis of laser capture microdissected breast tumors. Cancer Research (2017), 77 (13 Supplement); 213.
273. Stamler JS. Redox signaling: nitrosylation and related targetinteractions of nitric oxide. Cell (1994), 78(6); 931–6.
274. Su HY, Cheng W. Increased milk yield in transgenic mice expressing insulin-like growth factor 1. Animal Biotechnology (2004), 15(1); 9–19.
275. Su, S, Li, X., Li, S. Rutin protects against lipopolysaccharide-induced mastitis by inhibiting the activation of the NF-κB signaling pathway and attenuating endoplasmic reticulum stress. Inflammopharmacology (2018).
276. Sudlow A.W., Wilde C.J., Burgoyne R.D. Transforming growthfactor-h1 inhibits casein secretion from differentiating mammary-gland explants but not from lactating mammary cells. Biochemistry Journal (1994), 304; 333–336
277. Suszkiw J. New Mastitis Treatment May Offer Alternative to Antibiotics. Agricultural Research (2002), pg. 18
278. Talwar H, Bauerfeld C, Bouhamdan M, Farshi P, Liu Y, Samavati L. MKP-1 negatively regulates LPS-mediated IL-1β production through p38 activation and HIF-1α expression. Cellular Signaling (2017), 34; 1-10
279. Talwar H, Bouhamdan M, Bauerfeld C, Talreja J, Aoidi R, Houde N, Charron J, Samavati L. MEK2 Negatively Regulates Lipopolysaccharide-Mediated IL-1b Production through HIF-1a Expression. The Journal of Immunology (2019), 202(6); 1815-1825
280. Tang EH, Leung FP, Huang Y, Feletou M So K, Man RY, Vanhoutte PM. Calcium and reactive oxygen species increase in endothelial cells in response to releasers of endothelium-derived contracting factor. British Journal of Pharmacology (2007), 151; 15-23
281. Toerien C, Cant J. Abundance and phosphorylation state of translation initiation factors in mammary glands of lactating and nonlactating dairy cows. Journal of Dairy Science (2007), 90(6); 2726–34.
282. Tsapournioti S, Mylonis I, Hatziefthimiou A, Ioannnou M, Stamatiou R, Koukoulis G, Simos G, Molyvdas PA, Paraskeva E. TNFa Induces Expression of HIF-1a mRNA and Protein but Inhibits Hypoxic Stimulation of HIF-1 Transcriptional Activity in Airway Smooth Muscle Cells. Journal Cellular Physiology (2013), 228; 1745–1753

283. Tsuchiya M, Koizumi Y, Hayashi S, Hanaoka M, Tokutate Y, Yonekura S. The Role of Unfolded Protein Response in Differentiation of Mammary Epithelial Cells. Biochemical and Biophysical Research Communications (2017), 484; 903-908.
284. Uejyo, T., Kuki, C., Oyama, S, Kumura H, Kobayashi K. Early down-regulation of milk production after weaning by pup removal and prior to involution in mouse mammary glands. Cell Tissue Research (2015), 359; 643.
285. Unsworth A, Anderson R, Britt K. Stromal fibroblasts and the immune microenvironment: Partners in mammary gland biology and pathology? Journal of Mammary Gland Biology and Neoplasia. (2014), 19(2); 169-182.
286. Vanderhaeghen W, Cerpentier T, Adriaensen C,Vicca J, Hermans K, Butaye P; Methicillin-resistant Staphylococcus aureus (MRSA) ST398 associated with clinical and subclinical mastitis in Belgian cows. Veterinary Microbiology (2010), 144; 166–171
287. van Vliet AR, Agostinis P. Mitochondria-Associated Membranes and ER Stress. Current Topics Microbiology and Immunology (2018), 414; 73-102.
288. Veltmaat JM, Mailleux AA, Thiery JP, Bellusci S. Mouse embryonic mammogenesis as a model for the molecular regulation of pattern formation. Differentiation (2003), 71; 1 -17.
289. Vilte DA, Larzábal M, Cataldi AA, Mercado EC. Bovine Colostrum Contains Immunoglobulin G Antibodies against Intimin, EspA, and EspB and Inhibits Hemolytic Activity Mediated by the Type Three Secretion System of Attaching and Effacing Escherichia coli. Clinical Vaccine Immunology (2008), 15(8); 1208-1213
290. Vincent D, Elkins A, Condina M, Exernieks V, Rochfort S. Quantitation and Identification of Major Milk Proteins got High-Throughput LC-ESI-Q-TOF MS Analyses. PLoSONE (2016), 11(10); e0163471.
291. Vordenbäumen S, Saenger T, Braukmann A, Tahan T, Bleck E, Jose J, Schneider M. (2016), Human casein alpha s1 induces proinflammatory cytokine expression in monocytic cells by TLR4 signaling. Molecular Nutrition and Food Research (2016), 60; 1079-1089.
292. Vrieling M, Boerhout EM, van Wigcheren GF, Koymans KJ, Mols-Vorstermans TG, de Haas CJ, Aerts, Piet C, Daemen IJJM, van Kessel KPM, Koets, AP, Rutten VPMG, Nuijten PJM, van Strijp JAG, Benedictus L. LukMF′ is the major secreted leukocidin of bovine Staphylococcus aureus and is produced in vivo during bovine mastitis Scientific Reports (2016), 6; 37759
293. Wada N. Lipopolysaccharide stimulates expression of osteoprotegerin and receptor activator of NF-kappa B ligand in periodontal ligament fibroblasts through the induction of interleukin-1 beta and tumor necrosis factor-alpha; Bone (2004), 35(3); 629 - 635
294. Walker NI, Bennett RE, Kerr JF. Cell death by apoptosis during involution of the lactating breast in mice and rats. American Journal of Anatomy (1989), 185; 19-32.
295. Wang JJ, Wei ZK, Zhang X, Wang YN, Fu YH, Yang ZT. Butyrate protects against disruption of the blood-milk barrier and moderates inflammatory

responses in a model of mastitis induced by lipopolysaccharide. British Journal of Pharmacology (2017), 174(21); 3811–3822
296. Wang Y, Zhang Y, Chi X, Ma X, Xu W, Shi F, Hu S. Anti-inflammatory mechanism of ginsenoside Rg1: Proteomic analysis of milk from goats with mastitis induced with lipopolysaccharide. International Immunopharmacology (2019), 71; 382-391
297. Wärri A, Cook KL, Hu R, Jin L, Zwart A, Soto-Pantoja DR, Liu J, Finkel T, Clarke R. Autophagy and unfolded protein response (UPR) regulate mammary gland involutionby restraining apoptosis-driven irreversible changes. Cell Death Discovery (2018), 4(40); 1-13
298. Wasson, K. Retrospective Analysis of Reproductive Performance of Pair-bred Compared with Trio-bred Mice. Journal of the American Association for Laboratory Animal Science (2017), 56(2); 190-193(4).
299. Weiss WP, Hogan JS. Effects of Dietary Vitamin C on Neutrophil Function and Responses to Intramammary Infusion of Lipopolysaccharide in Periparturient Dairy Cows. Journal of Dairy Science (2007), 90(2); 731-739
300. Wellnitz O, Wall SK, Saudenova M, Bruckmaier RM. Effect of intramammary administration of prednisolone on the blood-milk barrier during the immune response of the mammary gland to lipopolysaccharide. American Journal of Veterinary Research (2014), 75(6); 595-601
301. Wible RS, Tran QT, Fathima S, Sutter CH, Kensler TW, Sutter TR. Pharmacogenomics of Chemically Distinct Classes of Keap1-Nrf2 Activators Identify Common and Unique Gene, Protein, and Pathway Responses In Vivo. Molecular Pharmacology (2018), 93(4); 297-308
302. Wigerup C, Påhlman S, Bexell D. Therapeutic targeting of hypoxia and hypoxia-inducible factors in cancer. Pharmacology & Therapeutics (2016), 164; 152-169
303. Williams JM, Daniel CW. Mammary ductal elongation: Differentiation of myoepithelium and basal lamina during branching morphogenesis. Developmental Biology (1983), 97(2); 274-290.
304. Wilson DJ, Stewart KN, Sears PM. Effects of stage of lactation, production, parity and season on somatic cell counts in infected and uninfected dairy goats. Small Ruminant Research (1995), 16(2); 165-169
305. Wilson E, Butcher EC. CCL28 Controls Immunoglobulin (Ig)A Plasma Cell Accumulation in the Lactating Mammary Gland and IgA Antibody Transfer to the Neonate. Journal of Experimental Medicine (2004), 200(6); 805-809
306. Wink DA, Mitchell JB. Chemical biology of nitric oxide: insights into regulatory, cytotoxic, and cytoprotective mechanisms of nitric oxide. Free Radical Biology in Medicine (1998), 25(4–5); 434–56.
307. Wyant G, Abu-Remaileh M, Wolfson R, Danai L, Vander Heiden M, Sabatini D. mTORC1 Activator SLC38A9 Is Required to Efflux Essential Amino Acids from Lysosomes and Use Protein as a Nutrient. Cell (2017), 171; 642-654
308. Wysolmerski JJ, Philbrick WM, Dunbar ME, Lanske B, Kronenberg H, Broadus AE. Rescue of the parathyroid hormone-related protein knockout mouse demonstrates that parathyroid hormone-related protein is essential for mammary gland development. Development. (1998) 125; 1285–1294

309. Wyszomierski SL, Rosen JM. Cooperative Effects of STAT5 (signal transducer and activator of transcription 5) and C/EBP β (CCAAT/enhancer-binding protein-β) on β-casein gene transcription are mediated by the glucocorticoid receptor. Mol Endocrinol. 2001;15(2):228–40.
310. Xiao HB, Wang CR, Liu ZK, Wang JY. LPS induces pro-inflammatory response in mastitis mice and mammary epithelial cells: Possible involvement of NF-κB signaling and OPN. Pathologie Biologie (2015), 63(1); 11-16
311. Xu R, Nelson CM, Muschler JL, Veiseh M, Vonderhaar BK, Bissell MJ. Sustained activation of STAT5 is essential for chromatin remodeling and maintenance of mammary-specific function. Journal of Cell Biology (2009), 184(1); 57–66
312. Xue X, Zhao N, Yu H, Sun Y, Kang C, Huang Q, Sun H, Wang X, Li N. Discovery of novel inhibitors disrupting HIF-1α/von Hippel–Lindau interaction through shape-based screening and cascade docking. PeerJ (2016), 4; e2757
313. Yao Y, Brodie AMH, Davidson NE, Kensler TW, Zhou Q. Inhibition of estrogen signaling activates the NRF2 pathway in breast cancer. Breast Cancer Research and Treatment (2010), 124; 585
314. Yang FL, Li XS. Role of antioxidant vitamins and trace elements in mastitis in dairy cows. Journal of Advanced Veterinary and Animal Research (2015), 2(1); 1-9
315. Yang W, Li H, Cong X, Wang X, Jiang Z, Zhang Q, Qi X, Gao S, Cao R, Tian W. Baicalin attenuates lipopolysaccharide induced inflammation and apoptosis of cow mammary epithelial cells by regulating NF-κB and HSP72. International Immunopharmacology (2016), 40; 139-145
316. Yeligar SM, Machida K, Kalra VK. Ethanol-induced HO-1 and NQO1 Are Differentially Regulated by HIF-1and Nrf2 to Attenuate Inflammatory Cytokine Expression. The Journal of Biological Chemistry (2010), 285(46); 35359–35373
317. Yonekura S, Tsuchiya M, Tokutake Y, Mizusawa M, Nakano M, Miyaji M, Ishizaki H, Haga S. The Unfolded Protein Response is Involved in Both Differentiation and Apoptosis of Bovine Mammary Epithelial Cells. Journal of Dairy Science (2017), 101; 3568-3578
318. Younis S, Javed Q, Blumenberg M. Meta-Analysis of Transcriptional Responses to Mastitis-Causing Escherichia coli. PLoS ONE (2016), 11(3); e0148562
319. Yu, QC, Song W, Lai D, Zeng YA. A Novel Mammary Fat Pad Transplantation Technique to Visualize the Vessel Generation of Vascular Endothelial Stem Cells. Journal of Visual Experiments (2017), (126), e55795.
320. Zachut M, Kra G, Portnik Y, Shapiro F, and Silanikove N Milk glucose-6-phosphate dehydrogenase activity and glucose-6-phosphate are associated with oxidative stress and serve as indicators of energy balance in dairy cows. Royal Society of Chemisty (2016), 6, 65412-65417
321. Zhang H, Liu Y, Weng J, Usuda K, Fujii K, Watanabe G, Nagaoka K. "Decrease of lactogenic hormones induce epithelial-mesenchymal transition via TGFβ1 and arachidonic acid during mammary gland involution" (2017), 63(3); 325-332.

322. Zhang HY, Wang ZG, Lu XH, Kong XX, Wu FZ, Lin L, Tan X, Ye LB, Xiao J. Endoplasmic Reticulum Stress: Relevance and Therapeutics in Central Nervous System Diseases. Molecular Neurobiology (2015), 51(3); 1343–1352
323. Zhang LL, Zhang ZJ. Sestrin2 aggravates oxidative stress of neurons by decreasing the expression of Nrf2. European Review for Medical and Pharmacological Sciences (2018) 22; 3493-3501.
324. Zhang M, Chen D, Zhen Z, Ao J, Yuan X, Gao X. Annexin A2 positively regulates milk synthesis and proliferation of bovine mammary epithelial cells through the mTOR signaling pathway. Journal of Cell Physiology (2018), 233; 2464–2475.
325. Zhang S, Qi H, Wen X-P, Li P, Gao X-J, Ao J-X. The phosphorylation of Tudor-SN mediated by JNK is involved in the regulation of milk protein synthesis induced by prolactin in BMECs. Journal of Cell Physiology (2019), 234; 6077–6090.
326. Zhang X, Qiao G, Lu P. Modulation of fibroblast growth factor signaling is essential for mammary epithelial morphogenesis. PLoS One. 2014;9(4):e92735.
327. Zhang X, Qiao G, Lu P. Modulation of fibroblast growth factor signaling is essential for mammary epithelial morphogenesis. PLoS One. 2014;9(4):e92735.
328. Zhao FQ. Biology of Glucose Transport in the Mammary Gland. J Mammary Gland Biol Neoplasia (2014), 19;3-17.
329. Zhao FQ, Keating AF. Expression and regulation of glucose transporters in the bovine mammary gland. Journal of Dairy Science (2007); 90(1):E76–86.
330. Zhao FQ, Moseley MW, Tucker HA, Kennelly JJ. Regulation of glucose transporter gene expression in mammary gland, muscle and fat of lactating cow sby administration of bovine growth hormone and bovine growth hormone-releasing factor. Journal of Animal Science (1996), 74; 183–189.
331. Zhao X, Lacasse P. Mammary tissue damage during bovine mastitis: Causes and control, Journal of Animal Science (2008), 86(13); 57–65
332. Zhou W, Lo SC, Liu JH, Hannink M, Lubahn DB. ERRβ: A potent inhibitor of Nrf2 transcriptional activity. Molecular and Cellular Endocrinology (2007), 278(1-2); 52-62
333. Zinkernagel, A.S., Johnson, R.S. & Nizet V. Hypoxia inducible factor (HIF) function in innate immunity and infection. Journal of Molecular Medicine (2007), 85; 1339 –1346.
334. Zowalaty A, Li1 R, Chen W, Ye X. Seipin deficiency leads to increased endoplasmic reticulum stress and apoptosis in mammary gland alveolar epithelial cells during lactation. Biology of Reproduction (2018), 98(4); 570–578.
335. Zwick R, Rudolph M, Shook B, Holtrup B, Roth E, Lei V, Van Keymeulen A, Seewaldt V, Kwei S, Wysolmerski J, Rodeheffer M, Horsley V. Adipocyte hypertrophy and lipid dynamics underlie mammary gland remodeling after lactation. Nature Communications (2018), SP 3592VL9IS-1AB.